THE EFFECTS OF TWO FORMS OF SLOW-RELEASE INSULIN ON LACTATING DAIRY COW METABOLISM AND MILK COMPONENT PRODUCTION

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THE EFFECTS OF TWO FORMS OF SLOW-RELEASE INSULIN ON LACTATING DAIRY COW METABOLISM AND MILK COMPONENT PRODUCTION

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Milk protein is the most valuable milk component for which dairy producers receive payment. Despite its high value, altering milk protein composition and production in dairy cows by diet has been challenging, particularly as compared to altering milk fat. Improving the efficiency of conversion of dietary nitrogen into productive nitrogen (as milk protein) has both financial and environmental benefits to the dairy industry. Dietary strategies to improve milk protein yield have focused on amino acid requirements, metabolizable protein, and the interaction between dietary energy and protein. However, dietary interventions have had only moderate success in improving milk protein production in well-fed dairy cows.

Use of long-term hyperinsulinemic-euglycemic clamps in lactating cows has suggested that milk protein synthesis in dairy cows is not maximized under normal management conditions, as significant improvements in milk protein have been observed in this experimental context. The goal of the present research was to expand on these observations by administering slow-release insulins, from human medicine, to lactating dairy cows. The hypothesis was that administration of slow-release insulins, without provision of supplemental glucose, would increase milk protein production and alter mammary metabolism in lactating dairy cows.

In experiment one, two forms of slow-release insulin were tested in a dose response study. Both Humulin-N (H) and insulin glargine (L) exerted insulin-like effects in lactating dairy cows, evidenced by linear decreases of plasma glucose with

increasing dose of slow-release insulin. In experiment two, H and L were given twice daily for 10 days to evaluate their effects on milk component production. Milk fat and protein content were both increased by treatment with H and L, suggesting that these forms of slow-release insulin alter metabolism and milk component synthesis. In experiment three, L was used in a mammary metabolism study to examine its effects on mammary uptake and utilization of substrates for protein synthesis. Reduced uptake of both essential and nonessential amino acids was observed during treatment with L, while milk protein yield remained the same for the control and treatment periods. This suggests that L alters metabolism and increases amino acid efficiency of use within the cow.

BIOGRAPHICAL SKETCH

Laurie Ann Winkelman was born on May 15, 1981 to John and Dee Winkelman in Oconomowoc, WI. Along with her older sister, Jeannie, she was raised on the family's dairy farm, Twinkle-Hill Farm, in Watertown, WI and developed a passion for dairy cows and the dairy industry. Laurie graduated from Watertown High School in 1999 as valedictorian of her class. During high school, she spent most of her days in art classes and the art room, and this passion for drawing continues today. Her connection to the family dairy farm led Laurie to attend college at the University of Wisconsin-Madison where she double majored in Dairy Science and Agricultural Journalism, graduating in 2003. An avid dairy cattle judge, Laurie was high individual at both the National 4-H Dairy Judging Contest (2000) and the National Intercollegiate Dairy Judging Contest (2002), making her just the fourth person in history to win both contests. A summer internship with Hoard's Dairyman magazine in 2000 planted the seed for an enduring freelance writing relationship with the publication, for which she has authored more than 25 articles. Laurie completed her Master's degree at The Ohio State University in 2006 under the direction of Dr. Chris Reynolds. Three months before defending her M.S. thesis in December 2005, she began working full-time as the Dairy Program Specialist at Ohio State, where she coordinated statewide dairy youth programs, coached the State 4-H and Ohio State University dairy judging teams, and helped coordinate adult extension programs including the Tri-State Dairy Nutrition Conference. In January 2007, Laurie continued her education journey and moved to Ithaca, NY to pursue her Doctorate degree with Dr. Thomas Overton at Cornell University. She continued coaching dairy judging teams and assisted Dr. David Galton in coaching the Cornell dairy judging team for 4 years. Following completion of her Ph.D. in 2011, Laurie will move back to her roots of eastern Wisconsin and begin working in a full-time position with Vita-Plus Corporation as a dairy nutritionist and member of the technical services team.

Dedicated to my family for all of their love and support

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Though the research and work reported in this dissertation is the accumulation of knowledge and experience I have gained through the last four years at Cornell University, this body of work would not have been possible without the support, guidance, advice, and friendship of numerous people.

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LIST OF ABBREVIATIONS

AA	Amino acids
ADF	Acid detergent fiber
AV	Arteriovenous
BW	Body weight
CP	Crude protein
CV	Coefficient of variation
DIM	Days in milk
DMI	Dry matter intake
ECM	Energy corrected milk
EF	Elongation factor
ELISA	Enzyme-linked immunosorbant assay
FDA	Food and Drug Administration
IF	Initiation factor
IGF-I	Insulin-like growth factor-I
IU	International unit
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MUN	Milk urea nitrogen
N	Nitrogen
NCN	Noncasein nitrogen
NDF	Neutral detergent fiber
NEFA	Non-esterified fatty acid
NE_L	Net energy for lactation
NFC	Non-fiber carbohydrate
NPN	Nonprotein nitrogen
NRC	National Research Council
PUN	Plasma urea nitrogen
RF	Release factor
RIA	Radioimmunoassay
RUP	Rumen undegraded protein
SCC	Somatic cell count
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
TBS-T	Tris-buffered saline-Tween 20
TMR	Total mixed ration
TN	Total nitrogen

CHAPTER ONE: INTRODUCTION

Protein is the most valuable milk constituent in multiple-component pricing systems, receiving the largest dollar per unit price of all the milk components. At the end of 2009, milk protein was valued at $6.35/kg ($2.88/lb), compared to $3.42/kg ($1.55/lb) for milk fat. Though protein prices have come down from the highs in 2009, demand for milk protein still appears to be strong as per capita cheese consumption has shown steady growth over the last 20 years, with an average annual consumption of more than 71 kg of cheese per capita in 2008. The financial incentive for milk with higher protein content, as well as the growing consumer demand for milk protein highlights the need to gain a greater understanding of nitrogen (N) efficiency within the lactating dairy cow and how milk protein is synthesized within the mammary gland from available substrates.

The efficiency of converting dietary crude protein into milk protein output in the lactating ruminant commonly ranges from 25 to 30 percent (Bequette et al., 1998). The conversion of metabolizable protein into true milk protein is about 0.62 to 0.64 g of true milk protein per g of metabolizable protein (Metcalf et al., 2008). These levels of efficiency, particularly in light of rumen N loss, also represent an area of opportunity for the dairy industry. Improving N efficiency within the cow and across the rumen will help the industry to avoid costly N loss to the environment, as low N efficiency of use leads to greater losses of N in urine (Broderick, 2003). The release of other pollutants to the environment from dairy cows such as ammonia can also be reduced by improving the N economy of the lactating cow (Frank et al., 2002).

The following review of the literature will describe strategies to alter milk protein composition in lactating dairy cows. From feeding and dietary strategies to regulation of protein synthesis at the cellular level, there appears to be opportunity to increase milk protein yield in the modern dairy cow.

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CHAPTER TWO: REVIEW OF THE LITERATURE

DIETARY STRATEGIES TO INCREASE MILK PROTEIN YIELD

Improving milk protein production in the lactating dairy cow has been a challenge facing dairy producers, nutritionists, and dairy scientists for many years. Though milk fat can easily be manipulated by dietary intervention, milk protein has proven to be more challenging to alter in well-fed dairy cows. From a rumen perspective, lactating dairy cows are rather inefficient at converting dietary crude protein into productive N (Bequette et al., 1998), but are more efficient at converting metabolizable protein into milk protein (Metcalf et al., 2008). Dietary strategies to increase milk protein production in lactating dairy cows have focused on increasing the metabolizable protein available to the cow, exploring the amino acid requirements for milk production, and investigating the interaction of protein and energy supply on milk protein yield. The following discussion will briefly look at amino acid utilization by the mammary gland and then will expand with further information on dietary strategies to meet the amino acid needs of the mammary gland for milk protein synthesis.

Amino acid utilization by the mammary gland

The amount of amino acids available in the arterial blood supply to the mammary gland is dictated by splanchnic release, which is dependent on absorption from the gut and use of amino acids by the portal-drained viscera and liver. Though the utilization of amino acids by the mammary gland may be more or less related to diets and rations, the supply of amino acids to the mammary gland is certainly dependent on diet, absorption of amino acids by the gut, and catabolism and transfer of absorbed amino acids across the portal-drained viscera (Lapierre et al., 2006; Pacheco et al., 2006). Therefore, amino acid use by the mammary gland will be briefly discussed here before more in-depth discussion about dietary strategies to improve milk protein yield.

When considering uptake and utilization of amino acids by the mammary gland, amino acids are generally divided into three groups based on the ratio of uptake from the blood to output in milk proteins (Mepham, 1982). Group 1 consists of essential amino acids that exhibit stoichiometric transfer from blood to milk protein and includes methionine, histidine, phenylalanine, tyrosine, and tryptophan. Group 2 consists of the amino acids which are taken up from the blood in excess of the amount secreted in milk protein. Group 2 amino acids include arginine, lysine, and the branched-chain amino acids valine, leucine, and isoleucine. The Group 2 essential amino acids are used within the gland to supply N for synthesis of nonessential amino acids and are also used as sources of energy (Mepham, 1982). Also, these amino acids may also be used for regulation of protein synthesis and other metabolic processes (Escobar et al., 2006). Group 3 amino acids are taken up from the blood in quantities that are deficient relative to their output in milk protein and broadly include the nonessential amino acids.

Metabolizable protein supply

The supply of metabolizable protein available to the cow post-ruminally includes three components: ruminally-undegraded crude protein (RUP) originating from feeds, microbial protein synthesized in the rumen, and endogenous crude protein. According to the NRC (2001), "The goals of ruminant protein nutrition are to provide adequate amounts of rumen-degradable protein for optimal rumen efficiency and to obtain the desired animal productivity with a minimum amount of dietary crude protein." Thus, it becomes necessary to monitor degraded and undegraded protein in the rumen to predict and optimize cow performance.

In lactating dairy cows, improving the supply of metabolizable protein post-ruminally by increasing the supply of RUP has increased milk yield and milk protein yield in a number of studies (Blouin et al., 2002; Noftsger and St Pierre, 2003; Raggio

et al., 2004; Weiss et al., 2009a). However, in some cases, the improvements in milk yield and milk protein yield under conditions of excess metabolizable protein supply result in reduced efficiency of transfer of feed N and amino acids into milk (Raggio et al., 2004; Weiss et al., 2009b).

Microbial protein, synthesized in the rumen, is a major supply of metabolizable protein and amino acids to the lactating dairy cow (Clark et al., 1992). When high quality forages are fed, the contribution of microbial protein to the metabolizable protein pool may be 50 to 55% of the cow's requirement (Chalupa and Sniffen, 2010). The amount of predicted metabolizable protein arising from microbial protein sources decreases when poor quality forages and silages are fed (Chalupa and Sniffen, 2010). In addition to overall forage quality, rumen microbial growth is dependent on the supply of ruminally-fermentable carbohydrate. Therefore, the end-products of rumen protein metabolism and passage of microbial protein to the duodenum are influenced by the source, degradability, and availability of carbohydrate supplies. Increasing the amount of grain in the diet can increase passage of microbial N to the duodenum (Oba and Allen, 2003). Sufficient fermentable carbohydrate available to the rumen microbial population is necessary to prevent losses of N as ammonia (Firkins et al., 2007). As microbial protein supplies a significant portion of protein available in the small intestine, predicting the passage quantity and quality of ruminally-synthesized microbial protein is of significant importance.

In practice, predicting microbial protein flow to the duodenum has not been simple and numerous approaches and models have been used to help gain a greater understanding of microbial protein flow out of the rumen (Firkins et al., 2006). Crude protein and ruminally-available protein supplied in the cow's diet serve as the N supplies for rumen microorganisms; therefore, it is important to have sufficient N available in the rumen for use by the microbes. The amino acid profile of microbial protein is thought to be relatively constant (Clark et al., 1992). The conversion of

microbial protein to metabolizable protein is around 64% (National Research Council, 2001; Metcalf et al., 2008).

Endogenous protein flow into the small intestine consists of scurf proteins and sloughed epithelial cells. Endogenous protein certainly contributes to the metabolizable protein pool in the small intestine and the Dairy NRC (2001) estimated that endogenous protein supplies about 5% of total protein to the cow. In a more recent review, researchers estimated the flow of free endogenous protein to the duodenum to be 13% (Marini et al., 2008). Though endogenous protein contributes to the metabolizable protein pool available to the cow as N, further discussion is beyond the scope of this review.

Amino acid nutrition

Research in dietary strategies to improve milk protein production has shifted from focusing on dietary crude protein concentrations to looking at amino acid supply and metabolizable protein that are available to the cow post-ruminally. As amino acids are the actual building blocks for protein synthesis, numerous research groups have attempted to determine amino acid requirements and limiting amino acids for milk protein synthesis in lactating dairy cows. Compared with monogastrics, determining the amino acid requirements of ruminants is much more complicated due to the effects of rumen fermentation and microbial protein supply.

Recent research has confirmed that, as a group, essential amino acids are indeed essential when metabolizable protein supplies are limiting (Doepel and Lapierre, 2010) and the mammary gland adjusts blood flow and uptake of amino acids under deficient conditions (Cant et al., 2001). Of the essential amino acids, lysine and methionine have been identified as the most limiting amino acids in lactating dairy cows (e.g. Schwab et al., 1976; National Research Council, 2001). Lysine appears to be either the first or co-limiting amino acid in early and at peak lactation (Schwab et

al., 1992a), whereas the status of methionine as the second-most limiting amino acid in that study was more variable. Determining the next most limiting amino acids beyond lysine and methionine has been less consistent, but phenylalanine (Nichols et al., 1998), valine (Broderick et al., 1974), histidine (Vanhatalo et al., 1999), and arginine (Mepham, 1982) may be limiting under varying dietary conditions.

A number of studies have been carried out to determine requirements for lysine and methionine in cows fed corn-based diets, and according to the NRC (2001) it was recommended that lysine contribute between 7 and 7.3% of metabolizable protein and that methionine contribute about 2.5% of metabolizable protein in diets for milk protein yield to be maximized (e.g. Schwab et al., 1992a; Schwab et al., 1992b; Rulquin et al., 1993; Pisulewski et al., 1996). In 2009, Schwab and coworkers reevaluated the breakpoint estimates reported in the NRC (2001) and found that for maximal milk protein content, lysine and methionine should comprise 6.80 and 2.29% of metabolizable protein, respectively. For maximal milk protein yield, the reevaluated lysine and methionine recommendations were more similar to the original NRC recommendations, consisting of 7.10 and 2.52% of metabolizable protein as lysine and methionine, respectively (Schwab et al., 2009). These recommendations may vary slightly depending on which ration balancing model is used, as slightly different approaches to predicting post-ruminal amino acid supplies in the models cause differences in the estimates for maximal yield of milk protein (Pacheco et al., 2006; Schwab and Foster, 2009; Whitehouse et al., 2009). The breakpoint estimates for optimal ratios of lysine to methionine in metabolizable protein for maximal milk protein yield are 2.82, 3.00, and 2.92 for the NRC (2001), CPM-Dairy (v.3.0.10), and AMTS.Cattle (v.2.1) models, respectively (Schwab et al., 2009).

Both lysine and methionine are commercially available in rumen-protected forms for use in dairy cattle rations. When diets are deficient in methionine, rumen-protected methionine can meet the methionine needs of the cow and improve

production (Patton, 2010). While other amino acids are not commercially available in rumen-protected form, the technology to protect amino acids from rumen degradation yet still allow digestion in the small intestine is available. Other rumen-protected amino acids have been used in research, such as rumen-protected arginine (Kirchgessner et al., 1993), though no positive effects of the arginine on milk production were observed in that study.

In a review by Firkins et al. (2006), the authors draw attention to the large variation associated with post-absorptive metabolism and the variation of success with either rumen-protected amino acids or increased dietary RUP. This variation, in combination with not having a full understanding of the amino acid requirements of the lactating dairy cow continue to provide challenges to scientists, dairy producers, and nutritionists in fine-tuning dairy cow rations.

The interaction of dietary protein and energy on milk protein

Dietary protein and energy concentrations have been extensively studied in an effort to improve milk protein production in lactating cows and have been the subject a number reviews (e.g. Sutton, 1989; Coulon and Remond, 1991). When either metabolizable protein or metabolizable energy are limiting, milk production and protein yield cannot be maximized. On the other hand, when either protein or energy are fed in excess, efficiency of use and conversion to milk energy is reduced, suggesting a point of marginal return in feeding energy and protein.

In a study by Broderick (2003), cows were fed increasing amounts of crude protein and increased amounts of dietary energy (through a reduction in dietary neutral detergent fiber [NDF]) in a large Latin square experimental design. There were no interactions between dietary crude protein and energy content for milk yield or milk protein yield, and the effects of increased crude protein in the diet on milk production were minimal. Cows fed higher crude protein diets had a significant and large decrease

in N efficiency as evidenced by large increases in milk urea and nonprotein N (NPN) and increased urinary N excretion. On the other hand, increasing the dietary energy concentration resulted in positive increases in milk yield and component yield while improving N efficiency and reducing milk NPN, urea N, and urinary N excretion.

In a study evaluating the interaction of energy and metabolizable protein in the diet, Rius et al. (2010b) also reported no interactions between diet energy and metabolizable protein concentration on milk yield or milk protein yield. Metcalf et al. (2008) suggested that metabolizable energy will only improve milk production when there is sufficient metabolizable protein; therefore, if metabolizable protein is limiting, additional metabolizable energy will not improve milk yield.

Other research has shown an interaction between dietary energy and protein on milk production and composition. Diets with low protein to energy ratios resulted in lower blood urea concentrations than diets with high protein to energy ratios (Brun-Lafleur et al., 2010). In that study, milk and protein yields increased more when dietary energy supply was increased in a high protein diet than in a diet designed to meet protein requirements. As alluded to in the previous paragraph, milk production and responses to either energy or protein are dependent on whichever nutrient is more limiting for production (Tedeschi et al., 2006).

Summary of dietary strategies to improve milk protein production

The Dairy NRC (2001) summarized available information regarding dietary influences on milk protein content and yield, with primary focus on modulation of milk protein through amino acid supplementation. In general, the opportunity to increase milk protein content and yield through known dietary strategies appears to be less than 5% per day. Though this review has only briefly discussed some of the dietary strategies to improve milk protein yield, it is likely that conventional approaches to increase milk protein will have modest success in well-fed dairy cows.

ALTERNATIVE STRATEGIES TO INCREASE MILK PROTEIN YIELD

Most of the protein and amino acid requirements discussed in the NRC (2001) and the research discussed above has focused on metabolizable protein and amino acids in the context of substrate supply for mammary milk protein synthesis. However a paradigm shift is occurring in dairy cattle research, and amino acids and energy supply are being investigated as regulators of the overall process of protein synthesis in addition to being used as substrates for the proteins themselves (Moshel et al., 2006; Toerien et al., 2010; Rius et al., 2010a). Focus on the regulation of protein synthesis may provide more information about how nutrients are used in the lactating cow mammary gland to synthesize the casein and whey proteins.

In addition to regulation of protein synthesis by nutrient supply, hormone signaling pathways can directly and indirectly affect the process of milk and milk protein synthesis, including the pathways of growth hormone (Hayashi et al., 2009), insulin (e.g. Park et al., 1979; Choi et al., 1988; Choi et al., 2004), prolactin (Choi et al., 1988; e.g. Choi et al., 2004), thyroid hormones (Miller and Swanson, 1969), insulin-like growth factor-I (IGF-I) (Cohick, 1998), steroid hormones, and leptin (Feuermann et al., 2008).

Insulin may be of key importance in milk protein synthesis because of its role in global protein synthesis and translation (rev. by Proud, 2006; Proud, 2007). Menzies (2009) investigated the role of insulin in mRNA expression of the major milk protein genes by culturing mammary explants with one of three treatments: no hormones, hydrocortisone and prolactin, and insulin, hydrocortisone, and prolactin. Maximal mRNA expression of α-S1 casein, α-S2 casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin, was observed with the culture that included insulin. Insulin also stimulates the release and secretion of proteins from mammary acini (Park et al., 1979).

Additionally, insulin and energy status (Campbell et al., 1999), and amino acids (Beugnet et al., 2003) are known to interact with and influence the mammalian

target of rapamycin (mTOR) signaling pathway (Wang et al., 2005). Microarray data from mammary explant work by Menzies et al. (2009) suggest that insulin stimulates mRNA expression of 28 genes directly related to protein synthesis and protein synthetic machinery. More detailed discussion of mTOR signaling and regulation of translational processes will follow.

There is evidence in lactating cows to support the hypothesis that insulin stimulates milk protein synthesis. Work with hyperinsulinemic-euglycemic clamps to study the effects of insulin in lactating dairy cows has shown significant increases in milk protein content and yield (McGuire et al., 1995; Griinari et al., 1997a; Mackle et al., 1999; Mackle et al., 2000). From this point forward, this review will focus on the role of insulin in milk protein synthesis, regulation of protein synthesis and translation, and potential methods to study the effect of insulin without provision of exogenous glucose supplies.

Hyperinsulinemic-euglycemic clamps

The use of hyperinsulinemic-euglycemic clamps in experimental contexts has been useful over the last 30 years as an in vivo model to examine whole body insulin response or action. First described in human subjects (DeFronzo et al., 1979), the concept of increasing circulating insulin concentrations by a constant insulin infusion rate with coinciding glucose infusion at a varied rate to maintain euglycemia has become the gold standard method for examining tissue insulin sensitivity. Hyperinsulinemic-euglycemic clamps have also been used to study the effects of insulin without the confounding effect of lowered glycemia or severe hypoglycemia.

The use of hyperinsulinemic-euglycemic clamps in animal experiments has grown over time, and one of the first experiments in large cattle was described by Sano et al., (1991) in which the clamp was used on lactating, late pregnant, and non-pregnant, non-lactating Japanese Shorthorn (beef) cows. During the short, two-hour

clamp period, a higher glucose infusion rate was required in lactating cows to maintain euglycemia as compared to non-lactating, late pregnant cows, suggesting differences in glucose uptake based on physiological state. Additionally, plateau insulin concentrations during infusion were lower in lactating and non-lactating, late pregnant cows compared to non-lactating, non-pregnant cows, indicating altered insulin uptake and utilization by physiological state.

With long-term hyperinsulinemic-euglycemic clamps carried out over days, rather than hours, numerous studies have shown increased milk protein content and yield in lactating dairy cows. With shorter duration of the hyperinsulinemic-euglycemic clamp (hours), changes in milk protein output have not been observed. An example of the plasma insulin and blood glucose concentrations observed during a hyperinsulinemic-euglycemic clamp in lactating cows is shown in Figure 2-1**.**

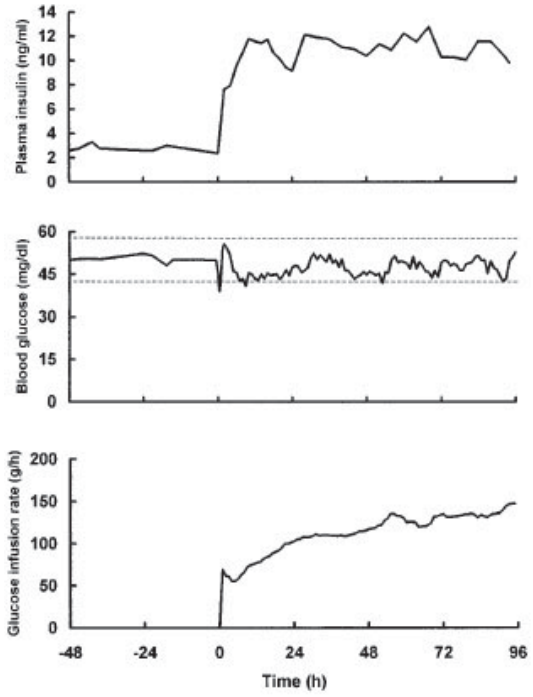

Figure 2-1. *Plasma insulin, blood glucose, and glucose infusion rate during a hyperinsulinemic-euglycemic clamp from Mackle et al. (1999). A constant infusion of insulin into jugular catheters was maintained for 4 d at a rate of 1 mg/kg of BW per hour. A 50% dextrose solution was co-infused with the insulin to maintain blood glucose concentrations within 10% of baseline concentration measurements taken before the start of the infusions.*

In lactating dairy cows, one of the first experiments using the hyperinsulinemic-euglycemic clamp technique was carried out to evaluate the effect of insulin on milk synthesis (McGuire et al., 1995b) and components of the IGF system (McGuire et al., 1995a). During the 4-d clamp period in which circulating insulin concentrations were elevated more than five-fold above baseline concentrations, McGuire and coworkers observed a 0.07 kg/d increase in milk protein yield, representing a 7% increase in milk protein output during the clamp period. In a subsequent experiment from the same group at Cornell University, Griinari et al., (1997a; 1997b) used the clamp technique in mid-lactation cows to elevate circulating insulin concentrations four-fold above baseline in conjunction with abomasal infusion of casein. During the clamp and abomasal infusion, milk protein yield was increased 0.23 kg/d or 28% above baseline production. With provision of additional protein substrate in the abomasum in the form of casein and amino acids, (Mackle et al., 1999) observed a 25% increase in milk protein output under hyperinsulinemic-euglycemic clamp conditions accompanied by a decrease in circulating essential amino acid concentrations, increased mammary blood flow, increased extraction efficiency of amino acids, and increased glucose uptake (Mackle et al., 2000). These results suggest that maximal milk protein production by the mammary gland has not been fully reached with common lactating rations. Additionally, it appears that the hyperinsulinemic-euglycemic clamp alters mammary gland uptake and metabolism of protein substrates.

Laarveld and coworkers (1981) used a co-infusion of insulin and glucose (not necessarily a hyperinsulinemic-euglycemic clamp, per se) to evaluate the effect of insulin on mammary arteriovenous differences in amino acids and metabolites across the mammary gland. They observed modest (10 to 20%) increases in uptake of some amino acids by the mammary gland with insulin treatment and increased extraction of aspartate, isoleucine, and leucine from the plasma.

The changes in milk protein content and yield observed under long-term hyperinsulinemic-euglycemic clamps provide insight into the hormonal regulation of mammary metabolism and protein synthesis. While the elevation of insulin often receives the credit for the change in milk protein content and yield (Griinari et al., 1997a; Mackle et al., 1999), the confounding effect of glucose infusion cannot be ignored.

One of the reasons the co-infusion of glucose during hyperinsulinemic-euglycemic clamps cannot be ignored in interpreting results is because of the substantial amount of energy that glucose provides to the cow during the clamp period. In the study by Mackle et al., (1999) the amount of glucose infused per day (3.336 kg) was the equivalent of 12.2 Mcal NE_L/d (Leonard and Block, 1997) which is a considerable amount of energy. As energy supply to the cow by diet exerts effects on milk protein content and yield (Broderick, 2003), the additional energy supply to the cow provided by infused glucose during hyperinsulinemic-euglycemic clamps may also be influencing mammary milk protein synthetic machinery.

Metcalf and coworkers (1991) attempted to carry out a close arterial infusion of insulin by infusing insulin directly into the external pudic artery on both sides of the mammary gland. The close arterial infusion of insulin resulted in a 36% increase in arterial plasma insulin concentrations. However, there was no change in arterial plasma glucose concentrations in this study, leading the authors to conclude that close arterial infusion of insulin in the mammary gland did not elevate systemic insulin concentrations. Large variation in blood flow complicated the results for this research group, and no changes in milk protein content were observed. This study is, however, an interesting approach to study the effect of insulin without co-infusing glucose.

The link between glucose, insulin, and milk protein production is also observed during glucose infusions. Amaral et al., (1990) observed a 10.5% increase in milk protein yield when cows were infused intravenously infused with 737 g/d of glucose.

A trend for higher plasma insulin in cows treated with intravenous (i.v.) glucose was observed in this study ($P = 0.12$, 499 vs. 634 pg/ml for control vs. i.v. glucose). Additionally, Rulquin et al., (2004) reported a quadratic increase in milk protein yield with duodenal glucose infusions, and this was associated with numerically higher arterial insulin concentrations. This study also showed that infusion of glucose caused the mammary gland to extract more essential amino acids than other treatments.

TRANSLATION AND PROTEIN SYNTHESIS

To understand how milk proteins are synthesized, it is useful to understand the basic process of mRNA translation and global protein synthesis. Eukaryotic protein synthesis and regulation of translation is fairly well understood and the subject is covered in numerous reviews (e.g. Rhoads, 1999; Jackson et al., 2010) and textbooks (e.g. Lodish et al., 2004). Recent research in dairy cows and bovine cell lines indicates that translation is a critical step in regulation of milk protein synthesis and is influenced by lactogenic hormones and amino acids (Toerien and Cant, 2007; Hayashi et al., 2009; Burgos et al., 2010). The three basic steps of mRNA translation which include initiation, elongation, and termination, will be discussed in the following section, along with the regulation of those steps by intracellular signaling cascades.

Translation initiation

The entire translation process is principally regulated at the initiation step (Jackson et al., 2010). Initiation is the most complex stage of translation and involves numerous protein-protein interactions and exchanges. Assembly of the ribosome occurs during initiation and is necessary for translation to begin. The ribosome consists of large and small ribosomal subunits, the 60S and 40S subunits. When the ribosomal subunits are not actively participating in translation, they are bound and kept apart by two initiation factors (IF), named eIF3 and eIF6 (Illustration 2-1).

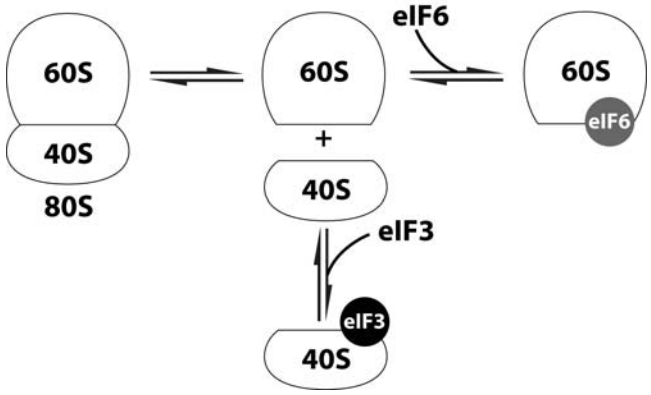

Illustration 2-1. *Simplified illustration of ribosome assembly adapted from Lodish et al (2004). The 80S ribosome, after completing a round of translation, dissociates into the 60S and 40S subunits which re-associate with their initiation factors, eIF6 and eIF3, respectively. These complexes are then recycled to undergo another round of translation.*

For the 40S ribosomal subunit to bind to the 60S subunit, a preinitiation complex must be formed between the 40S-eIF3 complex, another initiation factor, eIF1A, and a ternary complex consisting of an additional initiation factor (eIF2) which is bound with GTP and a methionine-charged tRNA (methionyl $tRNA_i^{Met}$) (Illustration 2-2, Step 1). An important control point of translation initiation is the phosphorylation status of the eIF2 bound to GDP. If eIF2 is phosphorylated, the eIF2-GDP complex cannot exchange bound GDP for GTP, inhibiting its ability to bind with the methionine-charged tRNA, which thus inhibits protein synthesis.

The mRNA awaiting translation is capped at the 5' end and is bound by an initiation factor, eIF4. The mRNA-eIF4 complex associates with the preinitiation complex (40S-eIF3-eIF1A-eIF2-GTP- methionyl $tRNA_i^{Met}$) and forms the initiation complex (Illustration 2-2, Step 2). The initiation complex scans the mRNA for the start codon. Upon reaching the start codon, the GTP associated with eIF2 is hydrolyzed (Illustration 2-2, Step 3), which is an irreversible step and prevents the initiation complex from any further scanning.

When the initiation complex reaches and associates with the start codon, the 60S ribosomal subunit joins the initiation complex to form the 80S ribosome. Another

initiation factor, eIF5, which is associated with GTP, is required to release eIF6 from the 60S subunit and free it to join the 40S subunit. Hydrolysis of the GTP associated with eIF5 subsequently frees the 60S subunit to associate with the 40S subunit, completing formation of the 80S ribosome (Illustration 2-2, Step 4).

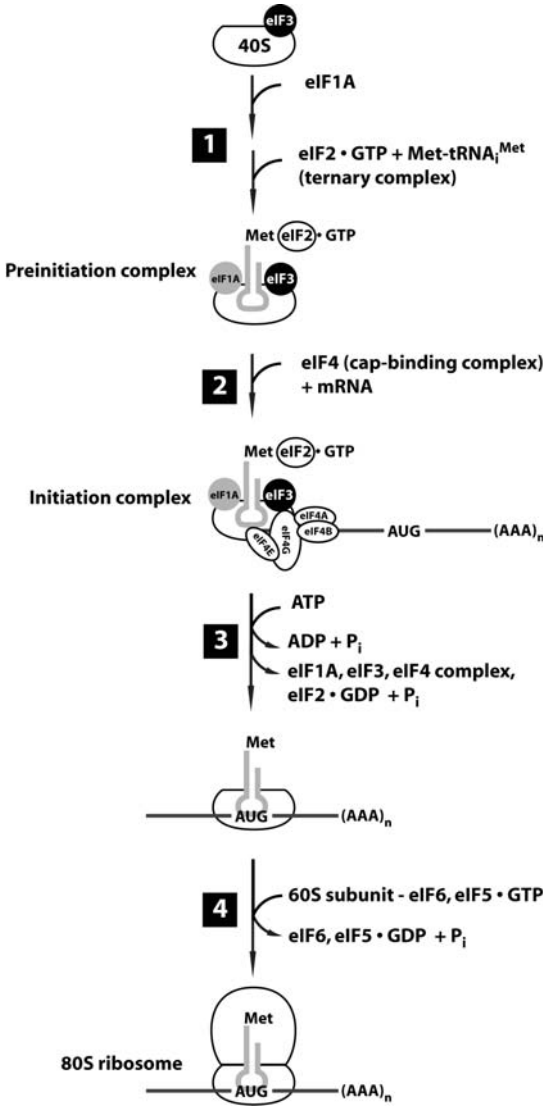

Illustration 2-2. *Initiation of translation and 80S ribosome assembly adapted from Lodish et al. (2004). The individual steps referenced in the text are shown in this figure as black squares containing numbers. Steps 1 and 2 indicate formation of the preinitiation and initiation complex through addition of various initiation factors. Step 3 illustrates the positioning of the 40S subunit after the initiation complex has scanned the mRNA for the start codon. Step 4 shows the release of initiation factors allowing the 60S subunit to join with the 40S subunit to form the 80S ribosome and start mRNA translation.*

Translation elongation

Upon correct positioning of the 80S ribosome, peptidyl chain elongation can begin. Another set of proteins is necessary for elongation to occur and these proteins are called elongation factors or EFs. Elongation of the peptidyl chain has three key steps: entry of amino acid-charged tRNAs, peptide bond formation, and the translocation of the ribosome complex along the mRNA one codon at a time. Each new amino acid to be added to the polypeptide chain is transported to the 80S ribosome complexed as an aminoacyl-tRNA associated with eEF1A-GTP. When the amino acid is correctly lined up with the mRNA codon, the GTP undergoes hydrolysis and results in the freeing of eEF1A-GDP from the tRNA and peptide bond formation in the growing polypeptide chain. Once the peptide bond is formed, the 80S ribosome must translocate down the mRNA. Translocation is promoted by the hydrolysis of eEF2-GTP to eEF2-GDP.

Translation termination

Upon reaching a stop codon on the mRNA strand, two release factors (RF) are involved in termination of translation and release of the ribosome from the mRNA. Release factor eRF1 and eRF3-GTP promote cleavage of the peptidyl-tRNA and release the completed protein. Following release, the protein is able to fold into its native conformation, which is facilitated by chaperone proteins.

Regulation of signaling pathways influencing protein translation

Translation is regulated by signaling pathways that affect phosphorylation status and GTP-GDP hydrolysis of the initiation, elongation, and termination factors. Many signaling pathways intersect and overlap to regulate these aforementioned translation factors. One of the major signaling pathways that influences protein translation is the mTOR signaling cascade.

Regulation of translation initiation factors. As mentioned earlier, the phosphorylation status of eIF2 dictates whether or not translation can begin. If eIF2-GTP is phosphorylated, it can bind with the ternary complex and participate in translation initiation. However, if eIF2-GDP is phosphorylated, then translation initiation will not occur. Serine 51 is the amino acid residue on eIF2 that undergoes changes in phosphorylation status. Four mammalian kinases are known to phosphorylate eIF2a on Ser51 (Jackson et al., 2010).

The ability of eIF4 to interact with the mRNA is also affected by phosphorylation status. However, it is not direct phosphorylation of eIF4 that changes its ability to interact with the mRNA, rather, it is the phosphorylation status of binding proteins that interact with eIF4 that dictate its activity. As shown in the earlier Illustration 2-2, eIF4 is a multi-protein complex consisting of eIF4A, eIF4B, eIF4E, and eIF4G. Phosphorylation status of eIF4E binding proteins, also known as 4E-BPs, affects the association of eIF4E with the eIF4 complex. When hypo-phosphorylated, 4E-BP is bound with eIF4E, making it unavailable for association with the eIF4 multi-protein complex. On the other hand, when 4E-BP is hyper-phosphorylated, eIF4E is released and allowed to interact and associate with the eIF4 complex, allowing translation initiation to proceed. Hyper-phosphorylation of 4E-BP, also often referred to as 4E-BP1, is under the control of mTOR.

In addition to altered phosphorylation status of the initiation factors, the 40S ribosome contains a subunit protein called ribosomal protein S6, or rpS6. This phosphorylation of this protein is carried out by S6 kinase 1 (S6K1). In addition to 4E-BP1, S6K1 is the second main target for the kinase activity of mTOR. When S6K1 becomes hyper-phosphorylated, it becomes active and is able to phosphorylate target proteins, including rpS6. Phosphorylation of rpS6 is permissive to translation and its phosphorylation status is positively correlated with protein synthesis (Thomas et al., 1982).

Regulation of translation elongation factors. Though initiation is highly controlled and very important in rates of translation, the elongation step is also subject to regulation and control by phosphorylation events (Browne and Proud, 2002). The two main elongation factors, eEF1 and eEF2, are targets for phosphorylation by different kinases. These kinases include protein kinase A (PKA), protein kinase C (PKC), and other kinases involved in cell cycle control.

Phosphorylation of eEF2 can and does occur at various amino acids, and in general, phosphorylation inhibits its activity. A specific kinase for eEF2 was identified 1987 and this kinase is dependent on calcium and calmodulin (rev. by Browne and Proud, 2002) and is aptly named eEF2 kinase. At some amino acids of eEF2 kinase, phosphorylation activates the kinase, while at other amino acids, phosphorylation inhibits the kinase activity. These amino acid sites are acted on by numerous other kinases including S6K1, PKA, AMPK and others (Illustration 2-3). As S6K1 is phosphorylated by mTOR, it becomes active and is able to phosphorylate eEF2 kinase. This phosphorylation of eEF2 kinase inhibits its activity. If eEF2 kinase is inhibited, then its downstream target protein, eEF2, is not being phosphorylated. Therefore, a downregulation in the phosphorylation of eEF2 is permissive to translation elongation. Indeed, insulin decreases the activity of eEF2 kinase and insulin also causes a decrease in the phosphorylation status of eEF2, which allows it to participate in elongation (Redpath et al., 1996).

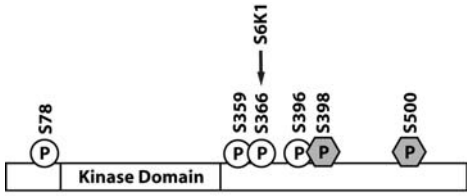

Illustration 2-3. *Sites of phosphorylation changes on eEF2 kinase, adapted from a review by Proud (2007). White circles represent sites that when phosphorylated inhibit kinase activity whereas gray hexagons are sites, that when phosphorylated, activate eEF2 kinase activity. The phosphorylation of eEF2 kinase by S6K1 at Ser 366 is inhibitory and under control of mTOR signaling pathways.*

Mammalian target of rapamycin signaling

The mammalian target of rapamycin, or mTOR, is a central mediator of cellular metabolism, cellular growth and apoptosis, and global protein synthesis (Yang et al., 2008). Numerous reviews have been published about mTOR and its role in these cellular processes (e.g. Proud, 2006; Proud, 2007; Yang et al., 2008). The mTOR signaling pathway is a crossroads for signaling cascades arising from various sources, including amino acids and insulin. Activation of the mTOR complex allows for downstream activation of proteins and kinases involved with the mRNA translation processes and proteins described earlier in this review. The mTOR complex contains multiple proteins including Ras homolog enriched in brain (Rheb, a GTP-GDP-bound protein), PRAS40, regulatory-associated protein of mTOR (raptor), and G protein β-subunit-like protein (GβL).

Nutrient status and availability impacts cellular metabolism and mRNA translation by affecting phosphorylation status and activation of multiple translation factors (Campbell et al., 1999). Insulin, upon binding to its cell surface receptor, causes recruitment and activation (by way of insulin receptor substrate, IRS) of phosphatidylinositol 3' kinase (PI3K). Upon activation, PI3K phosphorylates Akt at Thr 308 and Ser 473 (Zhang et al., 2009). When Akt is fully phosphorylated, it subsequently phosphorylates and inactivates the tuberous sclerosis complexes, TSC1 and TSC2. The tuberous sclerosis complexes inhibit mTOR activity. Once the repression of the TSC is removed by phosphorylation, mTOR enables the phosphorylation of 4E-BP1 and S6K1. Additionally, in vitro work has shown that PRAS40 is inhibitory to mTOR kinase activity, but phosphorylation of PRAS40 removes the inhibition on mTOR (Sancak et al., 2007). PRAS40 is a target of Akt phosphorylation. As mentioned earlier, both 4E-BP1 and S6K1 are important in regulating steps during translation. Illustration 2-4 shows the basic steps involved in insulin signaling and its interaction with mTOR.

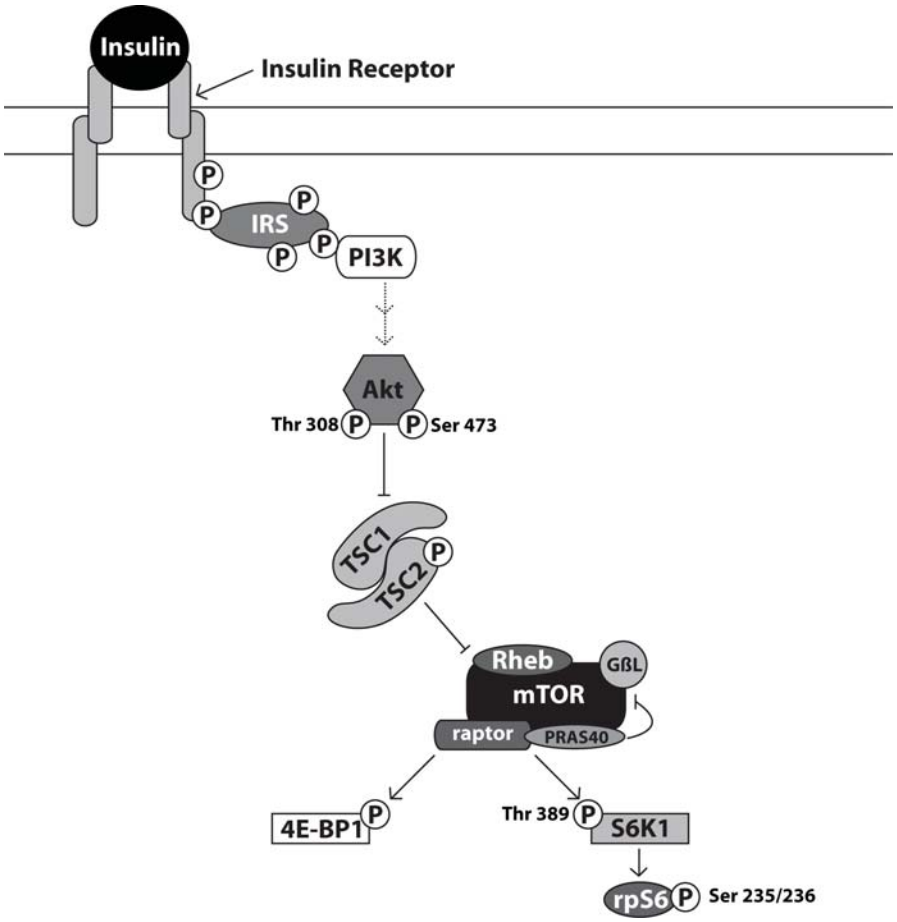

Illustration 2-4. *Insulin signal transduction pathway and its interaction with mTOR. Phosphorylation is represented in this illustration by encircled "P" and can represent phosphorylation at multiple amino acid sites. Insulin binds with its cell surface receptor resulting in recruitment and tyrosine phosphorylation of insulin receptor substrate (IRS). Phosphorylation of IRS results in activation of phosphatidylinositol 3' kinase (PI3K) which phosphorylates and activates Akt. Akt has kinase activity, which phosphorylates the tuberous sclerosis complexes (TSC). The TSC proteins inhibit mTOR activity by way of Rheb and phosphorylation of TSC removes the inhibition from mTOR. When active, mTOR phosphorylates 4E-BP1 and S6K1. These final steps are permissive to mRNA protein translation.*

There is evidence that the mTOR signaling pathway is at work in the mammary gland of lactating dairy cows. The research groups at the University of Guelph and Virginia Polytechnic University, as well as a few other universities, have looked at various proteins and kinases involved in mTOR signaling specific to bovines. Phoshorylation of rpS6 is greater in the mammary gland of lactating cows than non-lactating cows (Toerien and Cant, 2007). The level of mRNA expression of

proteins linking insulin and mTOR signaling are also increased by lactation including Akt, mTOR, eIF4E, and S6K1 (Bionaz and Loor, 2007). Additionally, mammary explants incubated with a combination of leptin, prolactin, and insulin showed increased expression of mTOR (Feuermann et al., 2008).

Burgos and Cant (2010) showed that global protein synthesis rates were increased by 47% in MAC-T cells treated with IGF-I. Responses to treatment of MAC-T cells with IGF-I included increased phosphorylation of Akt on Ser 473, a progressive increase in the phosphorylation state of S6K1, increased phosphorylation of 4E-BP1, and increased phosphorylation of TSC2. Additionally, in MAC-T cells, overexpression of eIF4E is associated with increased growth and proliferation of the cells (Long et al., 2001). All of the above responses correlate with higher translation and increased protein synthesis.

Toerien et al., (2010) used a feed deprivation-refeeding model in cows to study the effects of amino acids and glucose on protein synthesis. In this study, phosphorylation of S6K1 was higher for cows treated with essential AA and glucose compared to saline (control) cows. Additionally, the substrate for S6K1, rpS6, was also more phosphorylated for cows on this treatment compared to control cows. Though not statistically significant, insulin concentrations over the 9-h infusion period were numerically 29% higher for the essential amino acid and glucose treatment versus control. This study would represent acute changes in the protein synthetic machinery.

In research at Virginia Tech, abomasal starch infusion in intake-restricted cows increased circulating insulin concentrations (Rius et al., 2010a). In mammary tissue biopsies collected from these cows, there was an increased ratio of phosphorylated rpS6 to total rpS6 compared to control cows. These results suggest that insulin influences the mRNA translation process by altering phosphorylation status of kinases and proteins involved in protein synthesis.

In low producing cows, treatment with long-acting growth hormone stimulated milk yield and milk protein yield and affected the mTOR signaling cascade (Hayashi et al., 2009). In that study, phosphorylation of rpS6 was greater for cows treated with growth hormone and total abundance of eIF4E and eEF2 were greater in growth hormone-treated cows.

All of the above studies in lactating cows or bovine cell lines indicate that the mTOR signaling pathway is active within the mammary gland. Methods to influence and upregulate mTOR signaling in lactating cows warrant further investigation as a way to alter mammary milk protein synthesis.

INSULIN AND INSULIN ANALOGUES

The discovery and partially successful isolation of insulin extracts in the 1920s by Banting and Best was a scientific breakthrough that almost single-handedly took away the lethality of diabetes, which at the time, had been an incurable, untreatable disease (Bliss, 1982). Through a transition from animal insulins to recombinantly-made insulins, human diabetes has become a very treatable disease. Today, numerous insulins and insulin analogues are readily available for patients to use depending on type and severity of diabetes, time of day or timing of last meal, and other varying factors (Sheldon et al., 2009). Commercially available insulins and insulin analogues come in rapid-acting, intermediate-acting, and long-acting formulations.

An early form of insulin used in diabetes treatment included Neutral Protamine Hagedorn (NPH) insulin, which is a suspension of crystals of protaminated insulin. When combined with insulin in the suspension and injected into patients, the protamines slow the release of the insulin molecules into circulation. Introduced for commercial use in the 1940s, NPH is commonly classified as an intermediate-acting insulin. In its early uses, NPH insulin was used to replace basal insulin secretion in diabetic patients by administration once or twice per day. The peak action of NPH

insulin occurs 4 to 6 h after administration and effects can last between 12 and 16 h (rev. by Peterson, 2006).

One of the goals of insulin therapy is to reduce the risk of hypoglycemic episodes in patients while still maintaining good glycemic control. While NPH insulin was somewhat effective for overall glycemic control, there were still problems with hypoglycemic episodes. In an effort to obtain better control of glycemia, other insulin products with short or immediate action profiles were developed and introduced as mealtime insulins. Even with the development of mealtime insulins, there was still a need for better control of basal insulin concentrations. Eventually, a class of long-acting insulins were developed that provided better basal glycemic control and reduced the risk of hypoglycemic episodes. It should be noted, however, that although long-acting insulins are able to reduce the incidence of hypoglycemia, there is still considerable within-subject variability in response to insulin injection (Heinemann, 2002) which is of concern when treating diabetic patients.

Insulin glargine is a recombinant, human insulin analogue and is classified as a long-acting insulin, with up to 24 h in duration (Goykhman et al., 2009). Insulin glargine was approved for use in the United States by the Food and Drug Administration (FDA) in April 2000 and is sold under the trade name of "Lantus" by Sanofi-Aventis. Glargine differs from native human insulin with a glycine substitution for an asparagine residue at position A21 and addition of two arginine residues on the carboxy end of the B-chain at positions B31 and B32 (Bolli and Owens, 2000). The amino acid substitutions to this analogue shift the isoelectric point of the molecule from pH 5.4 to 6.8. The shift in the isoelectric point makes the analogue soluble in a more acidic solution than unaltered insulin, but when glargine is given subcutaneously, it precipitates at the more neutral pH of the injection site, slowly dissipating and being absorbed over a prolonged period of time. A comparison of the structures of human insulin, bovine insulin, and insulin glargine is depicted in Illustration 2-5.

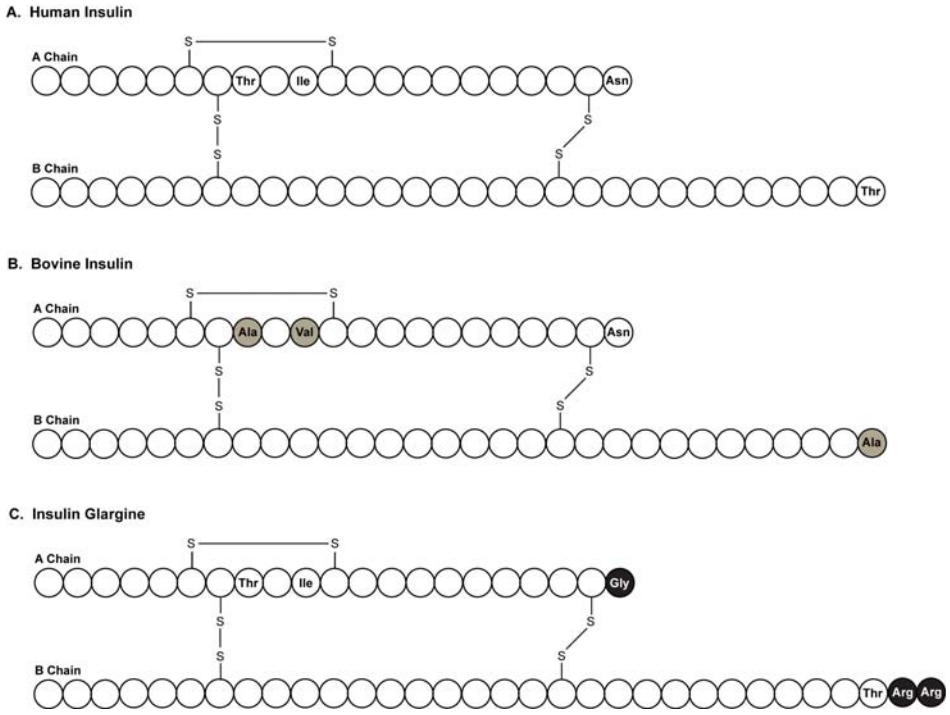

Illustration 2-5. *Comparison of the structures of human insulin (A), bovine insulin (B), and insulin glargine (C). Gray circles represent amino acid differences between bovine insulin and human insulin or insulin glargine. Black circles represent the amino acid substitutions and changes that are unique to insulin glargine.*

As opposed to intermediate-acting insulin formulations, such as NPH insulin, the pharmacokinetic profile of insulin glargine has a more peak-less action profile, thus mimicking basal insulin secretion more closely. In type 1 diabetic patients under isoglycemic clamps for 24-h, Lepore et al. (2000) observed an earlier onset of action for NPH insulin compared to insulin glargine, while the effect of insulin glargine lasted 8 h more than NPH insulin. Addtionally, in the study by Lepore and coworkers, insulin glargine had lower inter-subject variability than NPH insulin. Other forms of long-acting insulin such as insulin detemir are commercially-available for use in human medicine. However other specific analogues and their activities will not be discussed here as NPH insulin and insulin glargine are the main focus of interest in the present research.

Altering the structure of insulin impacts its affinity for insulin receptors, residence time on receptors, and affinity for other receptors such as IGF-I receptors. In

vitro studies using a variety of cell types have shown some interesting characteristics about insulin glargine activity. As NPH insulin is simply a protaminated form of regular insulin, the receptor affinity and pharmacodynamic characteristics of NPH insulin are not different from unaltered human insulin. Therefore, the following discussion will focus on these characteristics for insulin glargine only.

A study by Kurtzhals et al. (2000), examined the binding, metabolic, and mitogenic activities of 8 insulin formulations, including native human insulin and insulin glargine. In the human osteosarcoma cell line Saos/B10, insulin glargine exhibited nearly 8-times the mitogenic potency of normal human insulin. As was noted in that paper, the Saos/B10 cells express approximately 30,000 IGF-I receptors and less than 1,000 insulin receptors per cell. The abnormal ratio of IGF-I:insulin receptors may be very influential in the mitogenic responses observed in that particular study.

Other similar studies found that insulin glargine was only slightly more mitogenic than normal insulin. In using cardiac myoblasts and cardiomyocytes, Bahr et al., (1997) showed that insulin glargine had only slightly greater affinity for IGF-I receptors when compared to human insulin. In normal and transformed human breast epithelial cells, Staiger et al. (2007), measured DNA synthesis by ^{3}[H]thymidine incorporation in the presence of varying concentrations on insulin and insulin glargine. In the MCF-10 cells or normal breast epithelial cells, there were no statistically significant differences between insulin and insulin glargine at any concentrations tested for DNA synthesis. Similarly, in the transformed MCF-7 human breast carcinoma cells, there were no differences between insulin and insulin glargine at any of the concentrations tested.

Use of insulin and insulin analogues in lactating dairy cattle

The importance of insulin in metabolism and homeorhesis in lactating dairy cattle has been a focal point in numerous research groups over the years. Some dairy

cattle research has incorporated the use of altered insulin products to study the effects of insulin in cows. For example, Hayirli et al., (2002) used Humulin (Ultralente human insulin rDNA origin in extended zinc suspension) to evaluate effects of insulin on cow health and metabolism immediately postpartum. The insulin used in that study is no longer commercially available. However, the action profile of the Ultralente insulin would be somewhat similar to NPH insulin described earlier in this review, though the onset of action may be slightly later for Ultralente insulin and it may have higher insulin activity (Lepore et al., 2000). In the study by Hayirli and coworkers, cows receiving the lowest dose of slow-release insulin (0.14 IU/kg BW) by intramuscular injection appeared to have the most positive responses to treatment as evidenced by increased dry matter intake and milk yield, and reduced liver triglyceride. Milk composition was not reported for this experiment. In a brief review of the literature, it appears that protamine zinc insulins have been used less than a handful of times in dairy cattle, and most often this research has focused on the reduction of hepatic lipidosis and ketosis (e.g. Sakai et al., 1993; Hayirli et al., 2002). Additionally, the introduction of true long-acting insulins for use in humans has been a recent occurrence. Insulin glargine was approved for use in humans by the FDA in 2000 while insulin detemir received FDA approval in 2005. The pharmacokinetic and pharmacodynamic profiles of these analogues are substantially different than protamine insulins and could be used in dairy cattle research to elevate insulin activity.

RESEARCH OBJECTIVES

Continuing to fine tune dairy cow nutrition management to improve protein efficiency within the cow has large implications for both the environment and the bottom-line of dairy farms. As we continue to improve conversion efficiencies of feed nutrients and N into milk protein, less N will be wasted and excreted into the environment. Based on work with hyperinsulinemic-euglycemic clamps in lactating

dairy cows, it appears that milk protein synthesis under current management conditions is not maximized. While insulin is given the credit for boosting milk protein production in hyperinsulinemic-euglycemic clamp studies, the confounding effect of exogenous glucose, and thus energy, cannot be ignored. Ideally, the effects of insulin could be studied without large decreases in glycemia and the associated confounding effects of hypoglycemia.

In human medicine, insulin products and analogues exist that have extended duration of action, such as insulin glargine. The main hypothesis of the research presented within this dissertation is that slow-release insulins could be used in lactating dairy cows to study the effects of elevated insulin activity on milk protein synthesis, without the need to provide exogenous glucose to maintain relatively normal glycemia. As insulin affects and interacts with the mTOR signaling pathway, it is proposed that increased protein production will occur in cows treated with NPH insulin and insulin glargine, and that this will occur through stimulation of the insulin signaling pathway in the mammary gland, including phosphorylation and activation of Akt and mTOR, as evidenced by downstream phosphorylation changes in rpS6.

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CHAPTER THREE: RESPONSES OF LACTATING DAIRY COWS TO INCREASING DOSES OF TWO FORMS OF SLOW-RELEASE INSULIN

ABSTRACT

This study investigated the effect of increasing doses of two different types of slow-release insulin on the 24-h profiles of plasma glucose and insulin concentrations in lactating dairy cows. The study was carried out in two separately analyzed experiments investigating the effects of Humulin-N (H), a neutral protamine hagedorn insulin, and insulin glargine (L), an insulin analogue, at doses of 0 (control), 0.1, 0.2, and 0.4 IU/kg BW in a randomized complete block design. Sixteen cows were used for each insulin type, resulting in n = 4 for each dose within insulin type. Cows were fitted with a single jugular catheter on the day before the study. On the day of the study, cows were given treatments by subcutaneous injection of either sterile water or the designated insulin type and dose. Blood samples were taken hourly from the jugular catheter. Subcutaneous injection of both H and L resulted in linear decreases in plasma glucose concentrations ($P < 0.001$). Plasma insulin concentration linearly increased with increasing dose of H ($P < 0.001$). Though we were able to measure insulin in samples from cows treated with H, we were unable to obtain accurate insulin concentrations for samples from cows treated with L. We attempted to measure overall insulin concentrations and metabolites of L by a commercially-available ELISA and a commercially-available RIA kit, but did not retrieve values that we felt truly represent the amount of insulin activity being exhibited during this treatment. Though we were unable to measure actual insulin concentrations in cows treated with L, the observation of reduced plasma glucose in cows treated with L serves as a proxy for overall insulin activity. Both slow-release insulin products elicited insulin-like activity in lactating dairy cows, as evidenced by reduced plasma glucose concentrations. Given these results, the potential exists to use both H and L to study the effects of insulin

in lactating dairy cows without the confounding effect of severe hypoglycemia or concurrent provision of glucose during treatment.

INTRODUCTION

The use of hyperinsulinemic-euglycemic clamps in experimental contexts has been useful over the last 30 years as an in vivo model to examine whole-body insulin sensitivity. First described in human subjects (DeFronzo et al., 1979), the concept of increasing circulating insulin concentrations by a constant insulin infusion rate with concurrent glucose infusion at a varied rate to maintain euglycemia has become the gold standard method for examining whole-body insulin responsiveness.

In lactating dairy cows, one of the first experiments using the hyperinsulinemic-euglycemic clamp technique was carried out to evaluate the effect of insulin on milk synthesis (McGuire et al., 1995b) and components of the insulin-like growth factor system (McGuire et al., 1995a). During the 4-d clamp period in which circulating insulin concentrations were elevated more than five-fold above baseline concentrations, McGuire and coworkers observed a 0.07 kg/d increase in milk protein yield, representing a 7% increase in milk protein output during the clamp period. In a subsequent experiment from the same group at Cornell University, Griinari et al., (1997a; 1997b) used the clamp technique in mid-lactation cows to elevate circulating insulin concentrations four-fold above baseline in conjunction with abomasal infusion of casein. During the clamp and abomasal infusion, milk protein yield increased 0.23 kg/d or 28% above baseline production. With provision of additional protein substrate in the abomasum in the form of casein and amino acids, Mackle et al. (1999) observed a 25% increase in milk protein output under hyperinsulinemic-euglycemic clamp conditions accompanied by a decrease in circulating essential amino acid concentrations, increased mammary blood flow, increased extraction efficiency of amino acids, and increased glucose uptake (Mackle et al., 2000). These results suggest

that maximal milk protein production by the mammary gland has not been fully reached with common lactating rations in well-fed cows. Additionally, it appears that the hyperinsulinemic-euglycemic clamp alters mammary gland uptake and metabolism of protein substrates.

All of the studies referenced above indicate that insulin influences mammary gland protein synthesis. However, interpretation of results from hyperinsulinemic-euglycemic clamps is not that simple. The confounding effect of co-infusion of both insulin and exogenous glucose make it impossible to determine if the results are due to the effects of insulin, glucose, or both. During hyperinsulinemic-euglycemic clamps, significant amounts of glucose, and thus energy, are being infused into the cow. In the study by Mackle (1999), the amount of glucose infused per day (3.336 kg) was the equivalent of 12.2 Mcal NE_L/d (the NE_L value used for glucose calculation was described by Leonard and Block (1997) as 3.66 Mcal NE_L/kg), which is a considerable amount of energy. Additionally, infusion of glucose intravenously (Amaral et al., 1990) and into the duodenum (Rulquin et al., 2004) can also increase milk protein yield.

When administered by itself, insulin is commonly associated with reduced milk production and lowered blood glucose. Schmidt (1966) administered short-acting insulin subcutaneously in primiparous cows and observed an increase in milk protein percentage during insulin treatment, but no change in protein yield. Twice-daily injections of protamine zinc insulin in lactating dairy cows reduced total milk yield and blood glucose (Kronfeld et al., 1963). When given alone, the effect of insulin is also confounded by the effect of reduced glycemia. So, regardless of how the effects of insulin are investigated, either infused glucose or hypoglycemia may confound the results and interpretation. Ideally, the effects of insulin should be evaluated in situations without significant changes in glycemia or provision of additional glucose.

Numerous types of insulin and insulin analogues are used in human medicine to treat diabetes and their uses vary depending type and severity of diabetes, time of day or timing of last meal, and other varying factors (Sheldon et al., 2009). Commercially-available insulins and insulin analogues come in rapid-acting, intermediate-acting, and long-acting formulations. Insulin glargine is a recombinant, human insulin analogue and is classified as a long-acting insulin, lasting up to 24 h in duration (Goykhman et al., 2009). Insulin glargine differs from native human insulin with a glycine substitution for an asparagine residue at position A21 and addition of two arginine residues on the carboxy-end of the B-chain at positions B31 and B32 (Bolli and Owens, 2000). These amino acid substitutions to this analogue shift the isoelectric point of the molecule from pH 5.4 to 6.8 and make it more soluble in a more acidic solution. When insulin glargine is given subcutaneously, it precipitates at the more neutral pH of the injection site, slowly dissipating and being absorbed over a prolonged period of time. As opposed to other slow-release or intermediate-acting insulin formulations, such as neutral protamine hagedorn (NPH) insulins, the pharmacokinetic profile of insulin glargine has a more peak-less action profile, thus mimicking basal insulin secretion more closely. These characteristics make insulin glargine an option to explore the effects of elevated insulin activity in dairy cows without the need to infuse glucose to maintain euglycemia.

The use of long-acting or slow-release insulin in lactating dairy cattle has been limited. Hayirli et al. (2002) administered increasing doses of protamine zinc insulin (Humulin, Ultralente human insulin rDNA origin in extended zinc suspension) via intramuscular injection to early lactation cows on d 3 postpartum. Increasing dose of slow-release insulin in that study tended to increase milk yield in the early lactation cows, with cows receiving a dose of 0.14 IU/kg BW yielding 4 kg/d more from d 3 to 5 postpartum than controls. The quadratic effect of increased milk yield may have been due to a quadratic increase in dry matter intake (DMI). A once-daily injection

of 0.14 IU/kg BW resulted in serum insulin concentrations that were 1.75 times higher than the control in the first 24 h after injection. Meanwhile, circulating glucose concentrations were 5.2 mg/dl lower for the 0.14 IU dose. Effects of slow-release insulin on plasma glucose and insulin were diminished by 24 h after the first injection.

The objective of the present study was to evaluate the plasma profiles of glucose and insulin for 24 h following injection of increasing doses of two different forms of slow-release insulin. A NPH insulin (Humulin-N, 100 IU/ml human insulin, rDNA origin, Eli Lilly, Indianapolis, IN) was selected as one form of insulin in this experiment because of previous use of a similar type in dairy cows (Hayirli et al., 2002). Insulin glargine (Lantus, 100 IU/ml insulin glargine [rDNA origin], Sanofi-Aventis, Bridgewater, NJ) was selected based on its pharmacokinetic and pharmacodynamic characteristics.

MATERIALS AND METHODS

Animals, Experimental Design, and Treatments

The Cornell University Institutional Animal Care and Use Committee approved all procedures involving cows prior to the onset of the experiment. Thirty-two multiparous Holstein cows were adapted to a common TMR for 7 d before the day of treatment. Cows were divided into four groups containing eight cows each, based on DIM. Within each group, cows were randomly assigned to one of four treatments. Treatments included control (sterile water), low (0.1 IU/kg BW), medium (0.2 IU/kg BW), and high (0.4 IU/kg BW) doses of slow-release insulin. Cows were weighed once on the day before treatment commenced, after the morning milking and before daily feed delivery, to determine the total dose of slow-release insulin. For the first two groups of cows (237 ± 11 DIM, X ± SD), Humulin-N, (H; 100 IU/ml human insulin [rDNA origin] isophane suspension, Eli Lilly, Indianapolis, IN) was the form of slow-release insulin. For the second part of the experiment, insulin glargine, also known as

Lantus (L; 100 IU/ml insulin glargine [rDNA origin], Sanofi-Aventis, Bridgewater, NJ) was administered as the form of slow-release insulin to two groups of cows (213 ± 10 DIM, X ± SD).

On the day before treatment commenced in all groups, cows were fitted with an indwelling jugular catheter (Tygon Microbore Tubing, 1.778 mm o.d. x 1.016 mm i.d., Norton Performance Plastics, Akron, OH). On the day of treatment, two blood samples were collected prior to administration of either sterile water or slow-release insulin doses, and were used for covariate data in the statistical analysis. At 0900 h, cows were given treatments via subcutaneous injection in the neck. Blood samples were collected hourly for 24 h via jugular catheter and blood was transferred to tubes containing sodium heparin (30 U heparin/ml of blood). Blood samples were centrifuged (3,000 x *g* for 20 min at 4º C) and plasma was harvested into three aliquots, quickly frozen with liquid N, and then stored at -20º C until analysis for glucose and insulin.

A common TMR was fed to all cows ad libitum and was formulated to meet NRC nutrient recommendations (National Research Council, 2001). During the diet adaptation period, fresh feed was provided each morning at 0900 h, orts were weighed and recorded daily, and water was made available at all times. On the day of sampling, feed was delivered hourly to minimize fluctuations in circulating metabolites and hormones influenced by feed intake. Samples of the TMR were taken on the day of treatment and sent to Cumberland Valley Analytical Services (Hagerstown, MD) for analysis using wet chemistry techniques for CP (AOAC, 2000), acid detergent insoluble CP [CP determined using AOAC (2000) after acid detergent extraction (AOAC, 2000)], neutral detergent insoluble CP [AOAC (2000) method for CP after neutral detergent extraction (Van Soest et al., 1991)], soluble CP, (Krishnamoorthy et al., 1982), ADF (AOAC, 2000), NDF (AOAC, 2000), and ash (AOAC, 2000). Dietary ingredients and chemical composition of the basal

Table 3-1. *Dietary ingredients and nutrient composition of the basal diet (DM basis)*

Ingredient, %	Content
Corn silage	40.5
Alfalfa silage	16.1
Concentrate mix[1]	21.9
Corn meal	16.0
Corn distillers grain	5.5
Energy and nutrients[2]	
NE_L, Mcal/kg	1.68
CP	16.1
Soluble Protein, % CP	35.1
RDP, % CP	72.6
ADF	19.3
NDF	33.6
NFC	41.8
Ash	6.3
Calcium	0.70
Phosphorus	0.42
Magnesium	0.31
Potassium	1.27
Sodium	0.4
Iron, ppm	185.0
Manganese, ppm	62.7
Zinc, ppm	83.3
Copper, ppm	16.0

[1] The concentrate mix was comprised of (DM basis): 35.5% corn germ meal; 29.0% wheat midds, 12.6% canola meal, 5.6% calcium carbonate, 4.7% whey permeate, 3.9% blood meal, 2.5% sodium bicarbonate, 2.2% salt, 2.0% urea, 0.8% Optigen II (Alltech, Lexington, KY), 0.6% magnesium oxide, 0.2% calcium sulfate, 0.2% selenium 0.06%, 0.1% trace minerals, 0.1% Agrado (Novus International, Inc., St. Louis, MO), 0.1% vitamin A/D/E, and 0.1% Rumensin (Elanco, Indianapolis, IN).
[2] Values represent average values of TMR samples taken on each day of treatment.

TMR is reported in Table 3-1. Cows were milked three times daily at 0700, 1500, and 2300 h during the adaptation period and on the day of treatment and sample collection.

Plasma Hormone and Metabolite Analysis

Plasma concentrations of glucose were determined by enzymatic analysis (glucose oxidase) using commercially-available reagents (#P7119 PGO Enzyme Preparation, #F5803 3,3'Dimethoxy benzidine dihydrochloride; Sigma Aldrich, St. Louis, MO). Intra-assay and interassay coefficients of variation (CV) for glucose

analysis were 2.6 and 3.3%, respectively. Spectrophotometric readings were made using a $Versa_{max}$ tunable microplate reader (Molecular Devices, Sunnyvale, CA).

Plasma insulin concentrations were measured using two commercially-available kits. First, a bovine insulin ELISA kit reported to exhibit 28% cross-reactivity with insulin glargine (Mercodia Bovine Insulin ELISA #10-1201-01, Uppsala, Sweden) was used to measure the insulin values in the hourly plasma samples. Intra-assay and interassay CV for the ELISA were 4.3 and 6.4%, respectively. Plasma insulin concentrations were also determined by a commercially-available RIA kit which has 90% cross-reactivity with native bovine insulin (#PI-12K Porcine Insulin Radioimmunoassay, LINCO Research, St. Charles, MO). Intra-assay and interassay CV for the RIA insulin analysis were both 10%.

Statistical Analysis

Data from the H and L treatments were analyzed separately, as both treatments were not represented in all groups. With each type of slow-release insulin, data were analyzed using the MIXED procedure of SAS (2001). Fixed effects in the model included treatment dose, hour, and the interaction of dose and hour. Repeated measures for hour were included in the REPEATED statement. Group was included as a random effect in the model. Covariate data points collected before treatments commenced were used for covariate adjustment in the model.

RESULTS AND DISCUSSION

Sixteen cows completed the first part of the experiment utilizing H as the form of slow-release insulin. For the L portion of the experiment, one control cow was dropped from the experiment before treatment commenced due to a mobility issue unrelated to the experiment, resulting in 15 cows being used in the data analysis. On the day of treatment, DMI was not different between doses for cows given either type

of slow-release insulin. Dry matter intakes for cows treated with H were as follows: 27.9, 27.5, 29.4, and 29.0 ± 1.61 kg for the 0, 0.1, 0.2, and 0.4 doses, respectively (P = 0.83). For cows treated with L, DMI on the day of treatment were as follows: 29.1, 29.5, 28.3, and 25.7 ± 2.5 kg for the 0, 0.1, 0.2, and 0.4 doses respectively (P = 0.62). Though there was not an overall effect of treatment dose on DMI for the cows treated with L, the cows treated with the highest dose of L had numerically lower DMI, but there was no linear effect of L dose on DMI (Linear: P = 0.28). In ruminants, the effects of insulin on intake are variable. In a study designed to test the effect of insulin on long- and short-term feeding behavior, Baile and Mayer (1968) did not observe any changes in intake with insulin treatment and severe hypoglycemia. Insulin effects on DMI were more variable in sheep (Deetz et al., 1980), sometimes causing a decrease in intake while at other times having no effect on intake depending on dose or route of administration. Hayirli et al., (2002) observed a tendency for a quadratic effect of slow-release insulin on intake, with early lactation cows receiving a dose of 0.14 IU/kg having higher intakes than cows on other treatments. In the experiments by Kronfeld et al. (1963), numerous cows became recumbent and depressed during treatment with successive doses of insulin, and during these severe hypoglycemic episodes, the authors report that the cows did not eat. Thus it appears that insulin can have varied effects on ruminants, depending on the dosage rate and type of insulin administered. The overall goal of the present experiment is to determine if slow-release insulins can be used to elevate insulin activity without the need to infuse glucose to maintain glycemia within normal ranges. With the observation of a numerical decrease in intake at the highest dose of L, we are hesitant to use that dose in studies of longer duration as effects of severe hypoglycemia include anorexia.

Plasma glucose concentrations decreased linearly with increasing dose of slow-release insulin for cows administered both the H and L treatments. Plasma

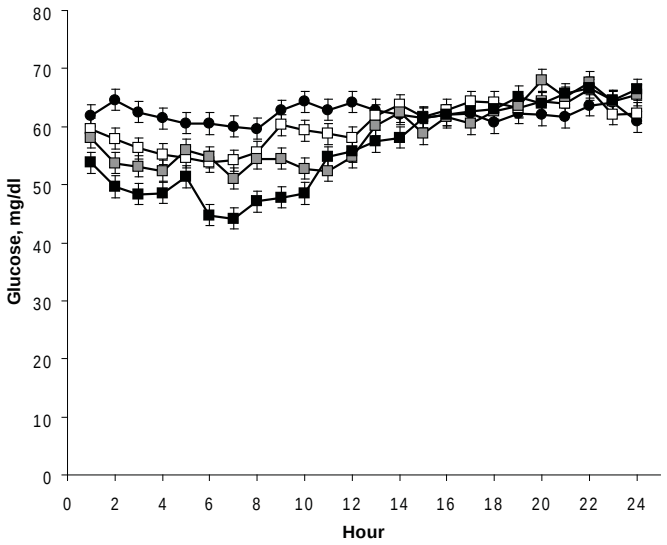

Figure 3-1. *Plasma glucose concentrations for cows injected subcutaneously with sterile water (–●–) or 0.1 (–□–), 0.2 (–▨–), or 0.4 (–■–) IU of Humulin-N per kg of BW. Probabilities of significance for effects of dose, hour, and dose x hour interaction were P < 0.001, P < 0.001, and P < 0.001, respectively. A linear decrease (P < 0.001) in plasma glucose was observed with increasing dose of Humulin-N treatment.*

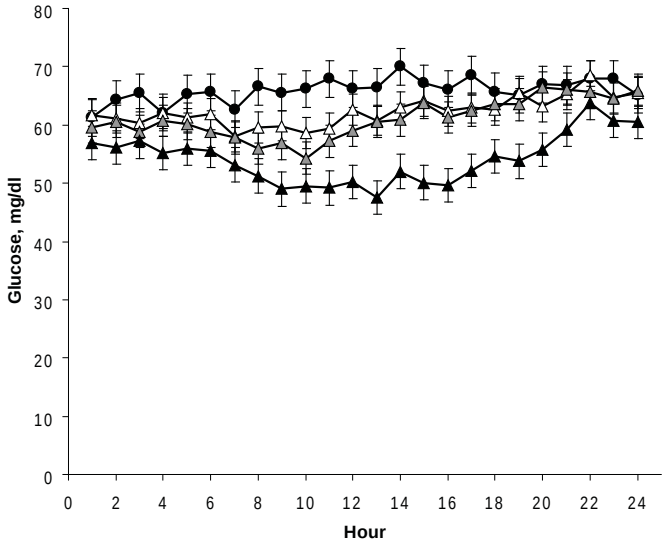

Figure 3-2. *Plasma glucose concentrations for cows injected subcutaneously with sterile water (–●–) or 0.1 (–△–), 0.2 (–◬–), or 0.4 (–▲–) IU of insulin glargine per kg of BW. Probabilities of significance for effects of dose, hour, and dose x hour interaction were P = 0.002, P = 0.002, and P = 0.32, respectively. A linear decrease (P < 0.001) in plasma glucose was observed with increasing dose of insulin glargine treatment.*

glucose responses to subcutaneous H and L injections are shown in Figures 3-1 and 3-2, respectively. Based on work with a similar slow-release insulin by Hayirli et al. (2002), it was expected that H would decrease plasma glucose concentrations in this experiment. Over the 24-h sampling period, plasma glucose concentrations were 3.1, 5.6, and 9.4% lower for the 0.1, 0.2, and 0.4 IU/kg BW treatments of H, respectively. Glucose concentrations were 62.1, 60.2, 58.7, and 56.3 ± 0.74 mg/dl for the control, 0.1, 0.2, and 0.4 IU/kg BW treatments of H, respectively (effect of dose: $P < 0.001$; linear: $P < 0.001$). Though the reductions in plasma glucose observed in this study were not as large as the decreases observed by Hayirli et al. (2002), it should be noted that the type of slow-release insulin used in that study was not exactly the same as the type used in this study. Additionally, the cows used in the present study were in late lactation, as opposed to the early postpartum cows used in the experiment by Hayirli and coworkers. Over the course of the 24-h sampling period, the 95% confidence intervals for the mean plasma glucose concentrations were 60.7 – 63.6, 58.7 – 61.7, 57.2 – 60.2, and 54.9 – 57.8 mg/dl for the control, 0.1, 0.2, and 0.4 IU/kg BW treatments respectively. Plasma glucose reached the lowest concentrations near the 6 and 7 h timepoints following injection.

For the cows treated with subcutaneous injections of L, plasma glucose concentrations were 5.6, 7.5, and 17.9% lower than the controls for the 0.1, 0.2, and 0.4 IU/kg BW doses, respectively (effect of dose: $P < 0.001$; linear: $P < 0.001$). Over the 24-h sampling period, glucose concentrations were 66.0, 62.3, 61.0, and 54.1 ± 1.94 mg/dl for the control, 0.1, 0.2, and 0.4 IU/kg BW treatments of L, respectively. Over the course of the 24-h sampling period, the 95% confidence intervals for the mean plasma glucose concentrations were 62.0 – 70.0, 59.3 – 65.2, 57.6 – 64.4, and 50.2 – 58.0 mg/dl for the control, 0.1, 0.2, and 0.4 IU/kg BW treatments respectively. Plasma glucose reached the lowest concentrations near the 12 h timepoint following injection. To our knowledge, L has never been administered to lactating dairy cows

or any ruminants. Though it was expected that L would exhibit insulin-like activity in dairy cows, we were unsure to what extent plasma glucose would be decreased following L injection.

As our goal of these experiments was to elevate insulin activity without the negative consequences resulting from severe hypoglycemia (e.g. anorexia, unconsciousness, disturbed behavior), we are hesitant to proceed the largest doses of either form of slow-release insulin with once-daily dose for longer term studies. During the 24-h sampling period, on the highest doses of both H and L, individual cows had plasma glucose concentrations near 20 mg/dl, which is not within normal ranges for lactating dairy cows under normal management conditions. The 95% confidence intervals of the mean plasma glucose concentrations for cows treated with the highest doses of both H and L suggest that cows treated with these doses did indeed enter a hypoglycemic state at times, though no visual signs of severe hypoglycemia were observed.

The first insulin assay used to attempt to measure plasma insulin was the Mercodia Bovine Insulin ELISA (Mercodia, Uppsala, Sweden). The cross-reactivity data from Mercodia stated the ELISA exhibited 28% cross-reactivity with L. Many other insulin assays do not state cross-reactivity with L, so this ELISA seemed like a logical starting point for insulin analyses.

When plasma insulin was measured by the Mercodia Bovine Insulin ELISA, a linear increase in plasma insulin was observed with increasing dose of H (Figure 3-3). For cows treated with H, plasma insulin was increased by 12.1, 25.6, and 67.9% for the 0.1, 0.2, and 0.4 IU/kg BW doses, respectively (effect of dose: $P < 0.001$; linear: $P < 0.001$). Insulin concentrations were 1.30, 1.46, 1.64, and 2.19 ± 0.143 ng/ml for the control, 0.1, 0.2, and 0.4 IU/kg BW doses of H, respectively. On the other hand, when looking at the cows treated with L, the results from the ELISA suggest that the assay does not exhibit cross-reactivity with the insulin glargine metabolites.

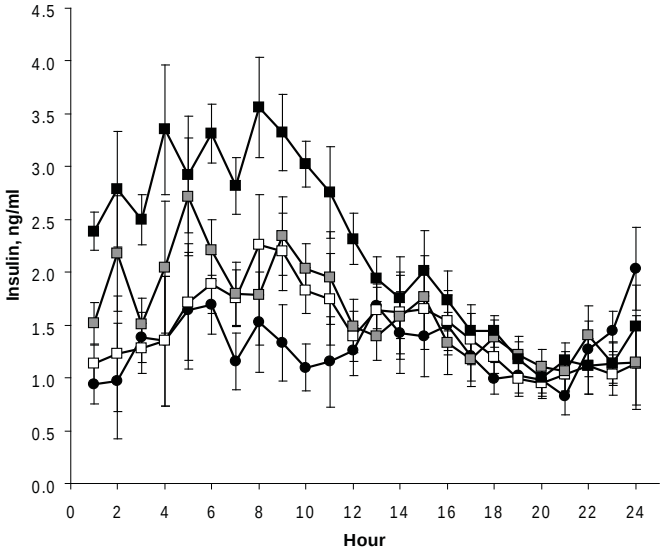

Figure 3-3. *Plasma insulin values, as measured by the Mercodia Bovine Insulin ELISA, for cows injected subcutaneously with sterile water (–●–) or 0.1 (–□–), 0.2 (–▣–), or 0.4 (–■–) IU of Humulin-N per kg of BW. Probabilities of significance for effects of dose, hour, and dose x hour interaction were P < 0.001, P < 0.001, and P = 0.29, respectively. A linear increase (P < 0.001) in plasma insulin was observed with increasing dose of Humulin-N treatment.*

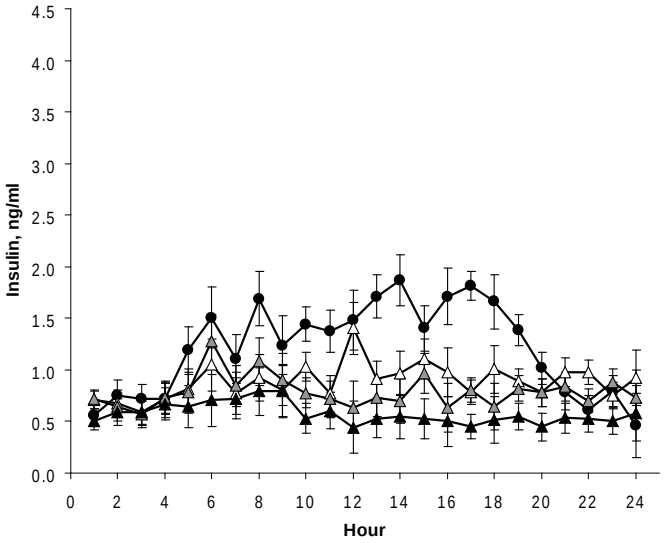

Figure 3-4. *Plasma insulin values, as measured by the Mercodia Bovine Insulin ELISA, for cows injected subcutaneously with sterile water (–●–) or 0.1 (–△–), 0.2 (–▲–), or 0.4 (–▲–) IU of insulin glargine per kg of BW. Probabilities of significance for effects of dose, hour, and dose x hour interaction were P < 0.001, P = 0.002, and P = 0.012, respectively. A linear increase (P < 0.001) in ELISA insulin values was observed with increasing dose of insulin glargine treatment.*

Using unadjusted data from the ELISA, cows treated with increasing dose of L had linearly lower values in the ELISA (Figure 3-4) and the least square means for the 24-h sampling period were 1.21, 0.89, 0.79, and 0.58 ± 0.092 ng/ml for control, 0.1, 0.2, and 0.4 IU/kg BW doses of L, respectively (effect of dose: $P < 0.001$; linear: $P < 0.001$). Intuitively, if the ELISA did in fact cross-react with L then, even with reduced overall cross-reactivity, greater values from the ELISA would be expected with increasing doses of insulin glargine. This was not the case, suggesting a few plausible explanations. First, the ELISA may be detecting endogenous concentrations of native bovine insulin. With extra insulin-activity in the system, it would seem plausible that the pancreas would secrete less insulin. Exogenous insulin supplied to an in situ perfused pancreas suppressed endogenous insulin secretion (Stagner et al., 1986). The work by Stagner et al. (1986) supports the concept that endogenous insulin production would be down-regulated in the present study for cows treated with L. Alternatively, the insulin glargine metabolites in the plasma samples may interfere with the ELISA antibody's ability to bind with insulin. That is, as more insulin glargine metabolites are in circulation with increasing dose of L, there may be increasing interference in the assay leading to reduced values in the ELISA. Finally, in detailed work with human insulin assays and detection of insulin glargine, Agin et al., (2007) forced solubilization of the insulin glargine molecule into its metabolites before submitting it to the assays, thus mimicking the natural precipitation of insulin glargine that occurs as it adjusts to physiological pH. If the cross-reactivity of insulin glargine with the ELISA was tested on the insulin glargine molecule, as opposed to breaking it down into its metabolites and then testing the cross-reactivity, the results reported in the kit information may be inaccurate.

To try to obtain a better idea of how much insulin-like activity was present in the cows treated with L, another insulin assay was used. The results reported here represent unadjusted values from the porcine insulin RIA, and as previously noted

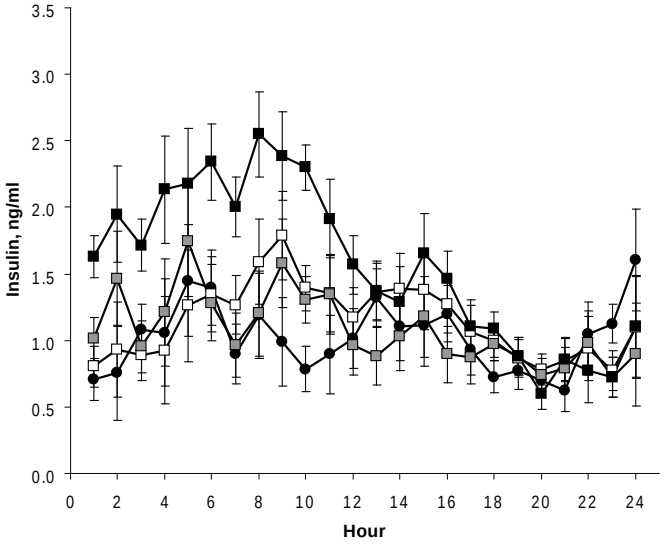

Figure 3-5. *Plasma insulin values, as measured by RIA, for cows injected subcutaneously with sterile water (–●–) or 0.1 (–□–), 0.2 (–▩–), or 0.4 (–■–) IU of Humulin-N per kg of BW. Probabilities of significance for effects of dose, hour, and dose x hour interaction were P < 0.001, P < 0.001, and P = 0.28, respectively. A linear increase (P < 0.001) in RIA insulin values was observed with increasing dose of Humulin-N treatment.*

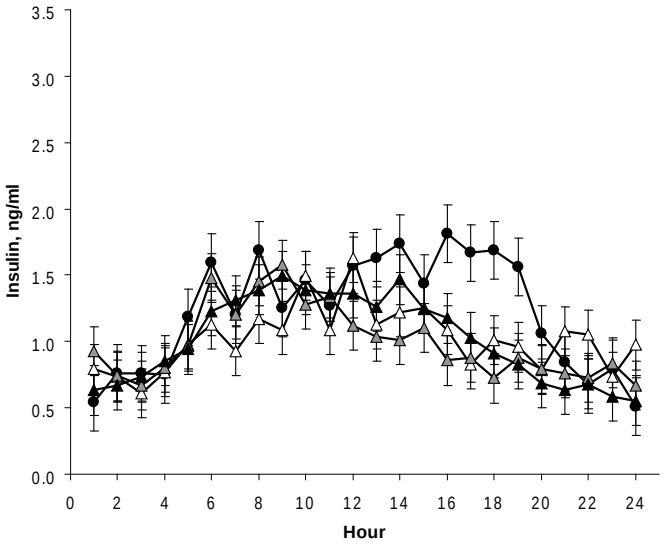

Figure 3-6. *Plasma insulin values, as measured by RIA, for cows injected subcutaneously with sterile water (–●–) or 0.1 (–△–), 0.2 (–◬–), or 0.4 (–▲–) IU of insulin glargine per kg of BW. Probabilities of significance for effects of dose, hour, and dose x hour interaction were P = 0.14, P < 0.001, and P = 0.23, respectively. A linear effect of dose (P = 0.07) on RIA insulin values was observed.*

this assay exhibits 90% cross-reactivity with bovine insulin. For cows treated with H, a linear increase ($P < 0.001$) in plasma insulin concentrations was observed with increasing dose of H (1.02, 1.15, 1.08, and 1.57 ± 0.097 ng/ml for control, 0.1, 0.2, and 0.4 IU/kg BW doses, respectively). The 24-h profile of insulin values for cows treated with H is shown in Figure 3-5. When results from the RIA were compared with those from the ELISA, the insulin values obtained with the RIA were between 21 and 34% lower than those obtained from the ELISA, keeping in mind that the RIA results were not corrected for the 90% cross-reactivity. Thus, with correction for cross-reactivity, the difference would become smaller.

For the cows treated with L, there was no overall effect of treatment for the values obtained from the RIA ($P = 0.14$; 1.22, 1.02, 0.99, and 1.02 ± 0.089 ng/ml for control, 0.1, 0.2, and 0.4 IU/kg BW treatments of L, respectively, with no adjustment for assay 90% cross-reactivity of the RIA). The 24-h profiles of insulin values

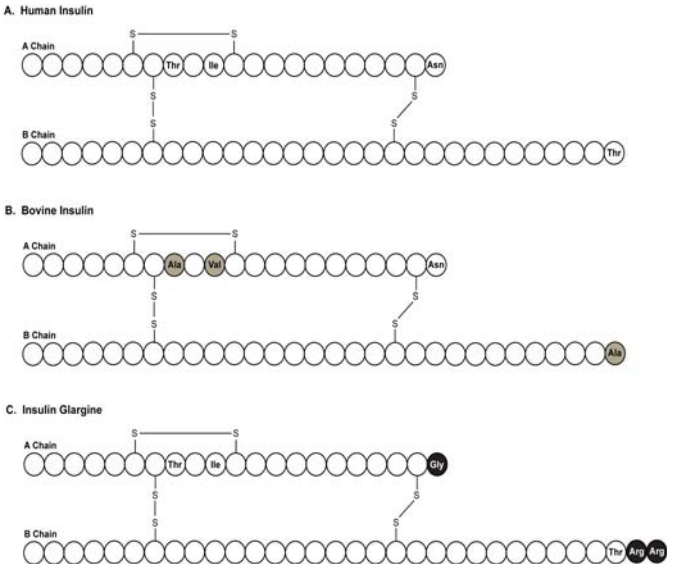

Figure 3-7. *Human insulin (A), bovine insulin (B), and insulin glargine (C) amino acid homology. Bovine insulin differs from human insulin by three amino acids, including an Ala substitution for Thr at amino acid 8 on the A chain and amino acid 30 on the B chain, and a Val substitution for Ile at amino acid 10 on the A chain. For insulin glargine, the base amino acid structure is similar to human insulin except for a Gly substitution for Asn on the carboxyl end of the A chain and the addition of two Arg residues on the carboxyl end of the B chain.*

obtained by RIA are shown in Figure 3-6 and there was a linear effect of L on insulin values for this assay ($P = 0.07$). Taken collectively with the results from the ELISA, it would seem plausible that the insulin glargine metabolites interfere with the antibody binding in the ELISA, thus showing reduced values in the assay for cows treated with increasing doses of L.

In selection of the assays used to measure insulin-activity following injection with slow-release insulins, the cross-reactivity of the two slow-release insulins to the antibodies in the assays was considered. Humulin-N is produced by a laboratory strain of *Escherichia coli* that has been genetically altered to produce human insulin, making the structure of H homologous to human insulin. As human insulin and bovine insulin are different by only three amino acids (Figure 3-7), depending on epitope location on the antibody, commercially-available insulin assays should be able measure H in plasma.

On the other hand, L has important structural modifications (Figure 3-7) that alter its ability to be detected by commercially-available insulin assays. When given subcutaneously, the insulin glargine molecule is solubilized into two metabolites, M1 and M2 (Kuerzel et al., 2003; Agin et al., 2007). These metabolites retain some of the same amino acid changes as the native glargine molecule. The cross-reactivity of L and its metabolites with antibodies to human insulin is lower than reactivity with native insulin. Immunoaffinity chromatography followed by liquid chromatography mass spectrometry can detect insulin glargine metabolites in urine (Thomas et al., 2007), however, no urine samples were taken during this set of experiments for that type of analysis. In later experiments, this method could be explored as a potential way to measure glargine metabolites in bovine urine.

Though we were unable to precisely measure insulin in the plasma samples taken from cows treated with L, our overall goal was to find a research model that could be used to extend the information learned from hyperinsulinemic-euglycemic clamps. Both slow-release insulin types reported here exhibited insulin activity in

lactating dairy cows, as evidenced by reduced plasma glucose concentrations during the 24-h period following injection.

During hyperinsulinemic-euglycemic clamps, oftentimes the goal is to maintain glycemia within 10% of baseline glucose concentrations (Mackle et al., 1999). In consideration of the data reported here, for all treatment doses and insulin types except the 0.4 IU/kg BW dose of insulin glargine, all mean plasma glucose concentrations were within 10 percent of the mean plasma glucose concentrations for the control treatment.

CONCLUSIONS

Both forms of slow-release insulin were effectively able to reduce circulating plasma glucose concentrations in lactating cows without severe hypoglycemia. By using slow-release insulins and eliminating the need for provision of additional glucose, there is potential to use these forms of insulin as an alternative to hyperinsulinemic-euglycemic clamps to investigate the effects of elevated insulin activity in lactating dairy cows. Though we were not able to exactly measure overall insulin concentrations in cows treated with insulin glargine, the response of reduced plasma glucose concentrations during treatment serves as a proxy for insulin activity within these cows.

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CHAPTER FOUR: EFFECTS OF SLOW-RELEASE INSULIN ON MILK AND COMPONENT PRODUCTION BY LACTATING DAIRY COWS

ABSTRACT

This study investigated the effect of two different types of slow-release insulin on milk production and composition in lactating dairy cows. Thirty multiparous cows averaging 88 days in milk were assigned to one of three treatments in a randomized complete block design. Treatments consisted of control (C), Humulin-N (H), and insulin glargine (L). The H and L treatments were administered twice daily at 12-h intervals via subcutaneous injection for 10 d. Cows were milked twice daily and milk composition was determined every other day. Mammary biopsies were performed on d 11 and mammary proteins extracted from the biopsies were analyzed by Western blot for components of insulin and mammalian target of rapamycin signaling pathways. There were no effects of treatment on DMI or milk yield ($P > 0.10$). Treatment with both forms of slow-release insulin increased milk protein content and milk protein yield was higher for cows treated with L. Milk fat content and yield were higher for cows treated with L compared with controls, but treatment with H did not affect milk fat content or yield. Lactose content was reduced by treatment with both slow-release insulins. Yields of milk casein were higher for cows treated with both forms of slow-release insulin, but the overall casein content as a percentage of true protein did not differ between treatment groups. Plasma glucose concentrations were lower for cows treated with both L and H compared to controls. Coinciding with the observation of higher protein yield, plasma urea nitrogen concentrations were lower for cows treated with L. Plasma NEFA concentrations were higher for cows treated with H as compared to control cows. Western blot analysis of mammary tissue collected by biopsy indicated that the ratio of phosphorylated Akt to total Akt differed by treatment ($P = 0.05$) and was greatest for cows treated with H. The ratio of phosphorylated

rpS6 to total rpS6 did not differ by treatment ($P = 0.60$). Modestly elevating insulin activity in lactating dairy cows by twice-daily subcutaneous injections of both H and L altered mammary metabolism and production of milk protein and fat. These forms of insulin may provide a model to study the effects of insulin without provision of exogenous glucose or severe hypoglycemia, but their effects on cell signaling within the mammary gland need further investigation.

INTRODUCTION

Insulin, a polypeptide hormone secreted by the β-cells of the islets of Langerhans in the pancreas, is a potent regulator of energetic metabolism and nutrient partitioning. Insulin exerts anabolic effects on tissues in both nonruminants and ruminants and acts to stimulate glucose uptake by muscle and adipose, stimulate glycogen synthesis, and also inhibit lipolysis, gluconeogenesis, and glycogenolysis. Although mammary uptake of glucose is insulin-independent (Laarveld et al., 1981; Zhao and Keating, 2007), insulin is still an important hormone in milk and milk component synthesis because of its roles in nutrient partitioning and energy metabolism (Brockman and Laarveld, 1986). Studying the effects of insulin in ruminants and dairy cows is a challenge because hypoglycemia can result when substantial doses are administered, resulting in decreased milk yield and dry matter intake (e.g. Kronfeld et al., 1963; Schmidt, 1966).

To overcome the confounding effects of hypoglycemia induced by administration of insulin, hyperinsulinemic-euglycemic clamps have been useful over the last 30 years as an in vivo model to examine whole body insulin sensitivity. First described in human subjects (DeFronzo et al., 1979), the concept of increasing circulating insulin concentrations using a constant insulin infusion rate with concurrent glucose infusion at a varied rate to maintain euglycemia has become the gold standard method for examining whole-body responses to insulin.

In lactating dairy cows, one of the first experiments using the hyperinsulinemic-euglycemic clamp technique was carried out to evaluate the effect of insulin on milk synthesis (McGuire et al., 1995b) and components of the insulin-like growth factor system (McGuire et al., 1995a). During the 4-d clamp period in which circulating insulin concentrations were elevated more than five-fold above baseline concentrations, McGuire and coworkers observed a 0.07 kg/d increase in milk protein yield, representing a 7% increase in milk protein output during the clamp period. In a subsequent experiment from the same group at Cornell University, Griinari et al., (1997a; 1997b) used the clamp technique in mid-lactation cows to elevate circulating insulin concentrations four-fold above baseline in conjunction with abomasal infusion of casein. The combination of insulin administration and casein infusion increased milk protein yield by 0.23 kg/d, or 28% above baseline. With provision of additional protein substrate in the abomasum in the form of casein and amino acids, (Mackle et al., 1999) observed a 25% increase in milk protein output under hyperinsulinemic-euglycemic clamp conditions that was accompanied by a decrease in circulating essential amino acid concentrations, increased mammary blood flow, increased extraction efficiency of amino acids, and increased glucose uptake (Mackle et al., 2000). These results suggest that maximal milk protein production by the mammary gland has not been fully reached within typical nutritional management. Furthermore, it appears that the hyperinsulinemic-euglycemic clamp alters mammary gland uptake and metabolism of protein substrates.

All of the studies referenced above indicate that insulin influences mammary gland protein synthesis. However, interpretation of results from hyperinsulinemic-euglycemic clamps is confounded by the co-infusion of both insulin and large amounts of exogenous glucose, which makes it difficult to determine if the results are due to the effects of insulin, glucose, or both. During hyperinsulinemic-euglycemic clamps, significant amounts of glucose, and thus energy, are being infused into the cow. In

the study by Mackle (1999), the amount of glucose infused per day (3.336 kg) was the equivalent of 12.2 Mcal NE_L/d (the NE_L value used for glucose calculation was described by Leonard and Block (1997) as 3.66 Mcal NE_L/kg), which is a considerable amount of energy.

When administered by itself, insulin is commonly associated with reduced milk production and lowered blood glucose. Schmidt (1966) administered short-acting insulin subcutaneously in primiparous cows and observed an increase in milk protein percentage during insulin treatment, but no change in protein yield. Twice-daily injections of protamine zinc insulin reduced milk yield and blood glucose in a study by Kronfeld et al. (1963). As previously mentioned, when given alone, the effect of insulin is also confounded by the effect of reduced glycemia. So, regardless of how the effects of insulin are investigated, either infused glucose or hypoglycemia may confound the results and interpretation. Ideally, the effects of insulin should be evaluated in situations without significant changes in glycemia or provision of additional glucose.

Numerous insulins and insulin analogues are readily available treatment of human diabetes depending on the type and severity of the diabetes, the time of day or timing of last meal, and other varying factors (Sheldon et al., 2009). Commercially-available insulins and insulin analogues come in rapid-acting, intermediate-acting, and long-acting formulations. Intermediate and long-acting insulins provide a more prolonged release of insulin following injection. These types of slow-release insulin have the potential to be used in dairy cows to study the effects of modestly elevated insulin activity without the negative effect of severe hypoglycemia.

The use of slow-release insulin in lactating dairy cattle has been limited. Hayirli et al. (2002) administered varying doses of protamine zinc insulin (Humulin, Ultralente human insulin rDNA origin in extended zinc suspension) via intramuscular injection to early lactation cows on d 3 postpartum. Increasing doses of slow-

release insulin tended to increase milk yield in the early lactation cows, with cows receiving a dose of 0.14 IU/kg BW yielding 4 kg/d more from d 3 to 5 postpartum than controls. The quadratic effect of increased milk yield may have been due to a quadratic increase in DMI. A once daily injection of 0.14 IU/kg BW resulted in serum insulin concentrations that were 1.75 times higher than the control in the first 24 h after injection. Meanwhile, circulating glucose concentrations were 5.2 mg/dl lower for the 0.14 dose. Effects of slow-release insulin on plasma glucose and insulin were diminished by 24 h after the first injection. In a previous study, we examined the 24-h profile of plasma glucose following a single subcutaneous injection of 0, 0.1, 0.2 or 0.4 IU/kg BW of either Humulin-N or insulin glargine in lactating dairy cows (Winkelman et al., 2010). Increasing doses of both forms of slow-release insulin caused linear decreases in plasma glucose concentrations and there were no effects on dry matter intake (DMI) during the day of study.

Insulin and other hormones have been reported to regulate initiation of protein synthesis in multiple cell culture systems and lactating dairy cow experiments (Hayashi et al., 2009; Toerien et al., 2010; Burgos and Cant, 2010; Burgos et al., 2010). Insulin may exert its effects on milk protein synthesis, observed in hyperinsulinemic-euglycemic clamp studies (e.g. Griinari et al., 1997a; Mackle et al., 1999), by activation of the mammalian target of rapamycin (mTOR) signaling cascade (Campbell et al., 1999; Proud, 2006; Proud, 2007). The roles of insulin and amino acids as they interact with mTOR signaling in the lactating dairy cow mammary gland has been examined to some extent (Rius et al., 2010), but are not fully understood.

The objective of the present study was to evaluate the effects of two different forms of slow-release insulin, Humulin-N and insulin glargine, on milk yield and composition by lactating dairy cows over a 10-d treatment period. It was hypothesized that milk protein content and yield would be increased by treatment with both types

of slow-release insulin. As Mackle et al., (1999) observed changes in milk protein production after 4 d using the hyperinsulinemic-euglycemic clamp, we expected that any changes in milk yield or composition would occur no later than 10 d after the start of treatments. Additionally, it was hypothesized that changes in milk protein and component synthesis would be the result of stimulation of the insulin and mTOR signaling pathways.

MATERIALS AND METHODS

Animals, Experimental Design, and Treatments

The Cornell University Institutional Animal Care and Use Committee approved all procedures involving animals prior to the onset of the experiment. Thirty multiparous Holstein cows ranging from 52 to 130 days in milk (DIM; 88 ± 25 DIM, X ± SD) at the onset of the experiment were blocked into two groups. Within each group, cows were assigned randomly to one of three treatments, with an aim to balance for DIM and average daily milk yield across all treatments prior to the start of the experiment. Treatments consisted of control (C), 0.2 IU/kg BW Humulin-N (H; 100 IU/ml human insulin [rDNA origin] isophane suspension, Eli Lilly, Indianapolis, IN), and 0.2 IU/kg BW Lantus (L; 100 IU/ml insulin glargine [rDNA origin], Sanofi-Aventis, Bridgewater, NJ). The H and L treatments were given 2x/day via subcutaneous injection, thus resulting in a total daily dose of 0.4 IU/kg BW for both treatments.

Beginning at least 7 d prior to the onset of the experiment, cows were fed a common total mixed ration (TMR) formulated using the Cornell Net Carbohydrate and Protein System version 6.1 (Tylutki et al., 2008) and designed to provide metabolizable protein and Met and Lys in excess of requirements (Table 4-1). Cows were offered a common TMR daily at 0900 h and orts were removed before feeding. Amounts of feed offered and refused were measured on a daily basis, and samples of the TMR

Table 4-1. *Dietary ingredients and nutrient composition of the basal diet (DM basis)*

Ingredient, %	Content
Corn silage	46.7
Ground corn	15.5
Wheat straw	6.9
Corn germ meal	5.2
Corn distillers	5.2
Canola meal	5.1
Amino Plus[1]	4.7
Minerals and vitamins[2]	3.0
Soybean meal	1.7
Blood meal	1.6
Citrus pulp, dry	1.6
Energy Booster[3]	1.1
Molasses	0.7
AminoShure-L[4]	0.5
Urea	0.3
Alimet[5]	0.1
Smartamine-M[6]	0.1
Energy and nutrients[7]	
NE_L, Mcal/kg	1.63
NDF	33.0
NFC	41.6
Starch	29.1
Crude fat	4.5
ME allowable milk,[8] kg/d	47.0
MP allowable milk,[8] kg/d	49.2
MP supply,[8] g/d	3,255
Lys,[8] % of MP	7.33
Met,[8] % of MP	2.54
CP	16.3
Lignin	2.7
Calcium	0.59
Phosphorus	0.39
Potassium	1.15
Magnesium	0.27

[1] Ag Processing, Inc., Omaha, NE
[2] Contained 2.80% Ca, 0.59% P, 0.49% S, 0.65% Mg, 1.40% K, 1.87% NaCl, 68 mg/kg of Cu, 6 mg/kg of I, 569 mg/kg Zn, 1 mg/kg Se, 30 IU/g Vitamin A, 116 IU/kg Vitamin E.
[3] Prilled saturated FFA, MS Specialty Nutrition, Dundee, IL.
[4] AminoShure L was a gift of Balchem Corporation, New Hampton, NY
[5] An 88% aqueous solution of dl, 2-hydroxy-4-(methylthio) butanoic acid, Novus International, Inc., St. Louis, MO
[6] Rumen-protected methionine (>70% DL-methionine, wt/wt), Adisseo USA, Alpharetta, GA.
[7] Values represent average of 3 weekly feed ingredient and TMR samples composited into one sample for analysis. The resulting analysis was inputted into the Cornell Net Carbohydrate and Protein System.
[8] Cornell Net Carbohydrate and Protein System predicted values based on feedstuff composition and a DMI of 26.26 kg/d.

were collected once each week and dried at 55°C until static weight was reached for measurement of dry matter (DM) content. Daily DMI were calculated for each cow using the respective amounts of feed consumed and the appropriate weekly DM content of the TMR. Samples of the TMR were sent to Cumberland Valley Analytical Services (Maugansville, MD) for analysis using wet chemistry techniques for crude protein (CP) (AOAC, 2000), acid detergent insoluble CP [CP determined using AOAC (2000) after acid detergent extraction (AOAC, 2000)], neutral detergent insoluble CP [AOAC (2000) method for CP after neutral detergent extraction (Van Soest et al., 1991)], soluble CP, (Krishnamoorthy et al., 1982), ADF (AOAC, 2000), NDF (AOAC, 2000), lignin (Goering and Van Soest, 1970), starch (Hall, 2009), sugar (Dubois et al., 1956), ether extract (AOAC, 2000), and ash (AOAC, 2000). Chemical composition of the basal TMR based on the composition of the components is reported in Table 4-1. Individual feed ingredients were also dried, sampled weekly, and analyzed in the same manner as the TMR described above. During the experiment, free choice water was available at all times.

Cows were weighed twice before the experiment commenced and the average weight was used to calculate daily dose of slow-release insulin. Covariate data and samples were collected prior to first treatment dose for all cows and included blood samples, DMI, and milk yield. Treatments were administered for 10 d at 12-h intervals beginning after milking on d 1. Blood samples were taken twice daily via coccygeal venipuncture into evacuated tubes containing sodium heparin (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) immediately prior to the 1000 h dose and again at 1600 h. Samples were placed on ice until centrifugation (3,000 x *g* for 20 min at 4ºC) and separation of plasma. Plasma samples were quickly frozen in liquid N and stored at -20ºC until further analysis.

Cows were milked twice daily at 0900 and 2100 h and milk yields were recorded at each milking. Milk samples were collected during each milking one day

prior to the onset of treatment and on d 2, 4, 6, 8 and 10 of the experiment and stored at 4°C with a preservative (2-bromo-2-nitro-1,3-propanediol) until analyzed (Dairy One Cooperative Inc., Ithaca, NY) for fat, true protein, and lactose using infrared analysis (AOAC, 2000; method 972.160), somatic cell count (SCC) by an optical fluorescent method (AOAC, 2000; method 978.26), and milk urea nitrogen (MUN). Energy-corrected milk (ECM) yield was calculated using the following equation from DHI: ECM, kg/d = (0.327 x milk kg) + (12.95 x fat kg) + (7.65 x protein kg) (Dairy Records Management Systems, 2011).

Additionally, composite milk samples from the covariate period, representing one day's milk by weighted volume, and from d 10 were analyzed for milk nitrogen fractions. Casein was determined by the indirect method (Lynch et al., 1998) such that casein was precipitated at pH 4.6 in acetic acid and sodium acetate, and noncasein N (NCN) in the filtrate was measured by Kjeldahl nitrogen analysis. Total nitrogen (TN) of the sample was also measured by Kjeldahl analysis (Barbano et al., 1990). The amount of casein (N x 6.38) in the sample was calculated as the difference between the amount of TN and NCN. To determine nonprotein nitrogen (NPN), proteins were precipitated and filtered from the sample by addition of 12% (wt.:vol.) trichloroacetic acid (Barbano et al., 1991). The nitrogen content of the filtrate, as measured by Kjeldahl nitrogen analysis, is the NPN (N x 6.38) fraction. The amount of true protein can be calculated by difference taking the difference between TN and NPN (N x 6.38).

Mammary Biopsies

On d 11, 12 to 15 h following the last treatment injection and 1 to 3 h after the morning milking, samples of mammary tissue were obtained by biopsy of the right hind quarter of all cows on the experiment. Xylazine was administered in the coccygeal vein (15 to 25 mg) to aid in animal handling during the procedure. The area to be biopsied on the right rear quarter of the mammary gland was aseptically

prepared with betadine and 70% ethyl alcohol. Local anesthetic (15 ml of 2% lidocane) was given under the skin at the biopsy site. A size 22 scalpel blade was used to cut an approximately 0.5 cm incision in the skin. A Bard Magnum trocar (12 gauge, 16 cm length) was inserted approximately 5 cm into the incision site reaching mammary tissue. The trocar was placed further into the mammary gland at three different sites and biopsy cores were obtained using the Bard Magnum Biopsy System and Bard TruGuide Coaxial Biopsy Needle (Bard, Covington, Georgia). Biopsy cores from each site were placed in aluminum foil packets, quickly frozen in liquid N, and stored at -80ºC until further analysis. After completion of biopsy core collection, sterile gauze was held on the biopsy site until bleeding ceased. If bleeding did not cease after pressure had been applied, super glue was used to close the incision site. Only 1 cow required the use of super glue to stop bleeding at the incision site. One cow developed mastitis after the biopsy due to difficulty in removing milk from the biopsied quarter. No noticeable declines in milk yield or DMI were observed in the remaining cows in the 7 d following the biopsy.

Mammary Protein Extraction and Western Blotting

Mammary proteins were extracted from the biopsy samples taken on d 11. Briefly, approximately 50 mg of tissue was homogenized on ice in 400 µl of cold lysis buffer containing 10 mmol/L Tris pH 7.5, 10 ml/L Triton X-100, 1 mmol/L EGTA, 150 mmol/L NaCl, and a protease and phosphatase inhibitor cocktail (#78441 Halt Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100X), Thermo Scientific, Rockford, IL). Protein concentration of the resulting supernatant was determined by the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology Inc., Rockford, IL). Supernatants were stored at -80ºC until electrophoresis.

Protein lysate supernatant was diluted in equal volumes with Laemmli SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10%

β-mercaptoethanol, and 0.004% bromophenol blue) and boiled for 5 minutes. Approximately 20 μg of protein was loaded onto 10% discontinuous polyacrylamide gels and electrophoretically separated until sample dye neared the bottom of the separating gels, approximately 90 minutes at 144 V (SE 260 Mighty Small Unit, Hoefer, Inc., Holliston, MA). Polyvinylidene fluoride (PVDF) membranes (Amersham Hybond-P, GE Healthcare, Fairfield, CT) were prepared for protein transfer by pre-wetting in 100% methanol for 1 to 2 seconds and then soaking in transfer buffer for approximately 15 minutes. Transfer cassettes were assembled and proteins were transferred from the gel to the pre-wetted membrane in a tank transfer system (TE 22 Mini Transfer Tank, Hoefer, Inc., Holliston, MA). Transfer was carried out at 22 V for 2 hours at room temperature. After transfer, membranes were rinsed with Tris-buffered saline containing 0.1% Tween (TBS-T) two times and non-specific binding sites were blocked for 1 h at room temperature using 2% (wt/vol) blocking reagent in TBS-T (ECL Advance Blocking Reagent, GE Healthcare, Fairfield, CT). Membranes were incubated with phospho-specific primary antibodies overnight at 4ºC. Membranes were washed 5x in TBS-T and incubated with secondary antibody in TBS-T containing 2% blocking reagent for 1 h at room temperature. Membranes were again washed 5x in TBS-T before incubation with chemiluminescence reagents.

Immunoreactive proteins were detected by enhanced chemiluminescence (Lumigen TMA-6, in the ECL Advance Western Blotting Kit, GE Healthcare, Fairfield, CT) by exposing the membranes to x-ray film (Amersham Hyperfilm ECL, GE Healthcare, Fairfield, CT), with varying exposure times to obtain detectable and measureable signal. The images on the film were analyzed for densitometry by NIH Image/J Software (http://rsb.info.nih.gov/ij/).

After detection of phospho-specific signals, the antibodies were stripped off of the membrane by incubation with stripping buffer (62.5 mM Tris-HCl, 100 mM

β-mercaptoethanol, and 2% SDS) at 55ºC for 30 minutes and washed with TBS-T before re-blocking and probing for total protein. All primary antibodies were obtained from Cell Signaling Technology (Beverly, MA) and were diluted as follows: anti-phospho-Akt (Ser 473) – 1:50,000, anti-Akt (total) – 1:20,000, anti-phospho-ribosomal protein S6 (rpS6) (Ser 235/236) – 1:50,000, anti-rpS6 (total) – 1:20,000. Secondary antibody (GE Healthcare, Fairfield, CT) was diluted 1:200,000. The densities of the phosphorylated signals were normalized to total protein levels.

Plasma Hormone and Metabolite Analysis

Plasma concentrations of glucose were determined by enzymatic analysis (glucose oxidase) using a commercially-available reagents (#P7119 PGO Enzyme Preparation, #F5803 3,3'Dimethoxy benzidine dihydrochloride; Sigma Aldrich, St. Louis, MO). Intra-assay and interassay coefficients of variation (CV) for glucose analysis were 2.6 and 3.3%, respectively. Plasma urea nitrogen was measured by the hypochlorite-phenol nitroprusside method using a commercially available enzymes and reagents (#U3383 Urease Buffer Reagent, #P6994 Phenol Nitroprusside solution, #A1727 Alkaline Hypochlorite solution; Sigma Aldrich, St. Louis, MO). Intra-assay and interassay coefficients of variation for PUN analysis were less than 4 and 7%, respectively. Additionally, plasma concentrations of NEFA were analyzed by enzymatic analysis (NEFA-C; Wako Pure Chemical Industries, Osaka, Japan). Intra-assay and interassay coefficients of variation for NEFA analysis were less than 3 and 5 percent, respectively. Spectrophotometric readings were made using a $Versa_{max}$ tunable microplate reader (Molecular Devices, Sunnyvale, CA). Plasma insulin concentration was determined by a commercially available radioimmunoassay (RIA) kit which has 90% cross-reactivity with native bovine insulin (#PI-12K Porcine Insulin Radioimmunoassay, LINCO Research, St. Charles, MO). Intra-assay and interassay CV for the RIA insulin analysis were both 10%.

Statistical Analysis

One cow was removed from the experiment on d 5 due to potential complications from insulin glargine treatment confounded by an injury to the left front leg. Thus data from d 1 to 4 represent 30 cows, while for d 5 through 10, only 29 cows are included in the data set. For plasma analysis, the MIXED procedure of SAS (SAS Institute, 2001) was used and included multivariate repeated measures to account for the nested sampling scheme of two plasma samples collected within each d for 10 d. The model included the main effects of treatment, day, time of sampling, and all of the associated two- and three-way interactions. For daily DMI and milk yield, the statistical model included the fixed effects of treatment, day, and the interaction of treatment x day. For the variables measured daily, multiple covariance structures were tested and the structure yielding the smallest Akaike's Information Criterion value was selected. For the tissue biopsy and western blot statistical analysis, the model included the effect of treatment. Protein yield prior to the start of treatments was included as a covariate. Pretreatment values for DMI, milk yield, and plasma variables measured or assessed during the week before assignment to treatments were used as covariates during analysis of covariance for their respective analyses.

RESULTS AND DISCUSSION

The nutrient composition of the TMR is presented in Table 4-1. As designed, the diet contained excess metabolizable protein and supplemental Met and Lys to ensure that enough substrate would be available for protein synthesis if the cows responded to slow-release insulin treatments. Based on mean DMI (Table 4-2) for each treatment and nutrient analyses of the diet ingredient feedstuffs, the Cornell Net Carboyhydrate and Protein System (v. 6.1) (Tylutki et al., 2008) predicted the available MP supply (unadjusted for maintenance MP requirements) to be 3,273 g/d for C, 3,247 g/d for cows treated with H, and 3,327 g/d for cows treated with L.

Table 4-2. *Effect of twice-daily subcutaneous injections of two types of slow-release insulin on dry matter intake, milk yield, and milk composition*

	Treatment[1]				*P*-value				
Variable	C	H	L	SEM[2]	Trt	Day	Trt x Day	C vs. H	C vs. L
DMI[3], kg/d	26.4	26.2	26.8	0.41	0.579	<0.001	0.569	0.748	0.484
Milk yield, kg/d	48.3	47.3	47.1	1.14	0.464	0.124	0.293	0.329	0.255
ECM[4], kg/d	46.8	46.5	48.3	1.12	0.502	0.080	0.623	0.836	0.362
Fat, %	3.17	3.32	3.50	0.110	0.118	0.083	0.278	0.357	0.041
Fat yield, kg/d	1.50	1.55	1.65	0.052	0.126	0.213	0.832	0.436	0.045
Protein, %	3.00	3.20	3.29	0.045	0.001	<0.001	0.416	0.004	<0.001
Protein yield, kg/d	1.46	1.49	1.54	0.030	0.084	0.001	0.076	0.277	0.028
Lactose, %	4.84	4.76	4.70	0.024	0.001	0.130	0.254	0.015	<0.001
Lactose yield, kg/d	2.34	2.26	2.21	0.039	0.068	0.036	0.063	0.130	0.023
Total solids, %	11.95	12.09	12.42	0.137	0.064	0.025	0.281	0.488	0.024
Total solids yield, kg/d	5.77	5.68	5.82	0.128	0.626	0.129	0.606	0.537	0.729
SCC (x 1,000)[5]	62	44	113	23.7	0.124	0.176	0.265	0.608	0.142
MUN[6], mg/dl	13.5	12.5	12.3	0.52	0.012	<0.001	0.076	0.017	0.006

[1] Treatments: C = control, H = 0.2 IU/kg BW Humulin-N, L = 0.2 IU/kg BW insulin glargine given twice daily via subcutaneous injection at 1000 h and 2200 h.
[2] SEM = standard error of the mean.
[3] DMI = dry matter intake.
[4] ECM = energy-corrected milk. ECM calculated as equal to [(0.327 x milk yield) + (12.95 x fat yield) + (7.65 x protein yield)].
[5] SCC = somatic cell count.
[6] MUN = milk urea nitrogen.

DMI, Milk Yield, and Milk Composition

Production results are presented in Table 4-2. Dry matter intake, milk yield, and ECM did not differ between treatments ($P > 0.05$). In multiple experiments, Kronfeld et al. (1963) administered protamine zinc insulin, another form of slow-release insulin, intramuscularly twice daily at various doses to cows. In one of the experiments, five successive doses of 0.24 U/kg, which was similar to the dose used in the present experiment, had little effect on plasma glucose or milk production. When the insulin dose was increased two-fold, both milk yield and plasma glucose concentrations were decreased markedly (Kronfeld et al., 1963). Though this part of the experiment by Kronfeld et al. (1963) only had one animal, it supports the observations of the present experiment showing moderate doses of slow-release insulin have little to no effect on milk yield. On the other hand, it appears that higher doses of insulin have greater impact on milk yield and dry matter intake. Cows given regular insulin at a dose of 0.3 U/kg BW (Schmidt, 1966) after each milking showed decreased milk production during the treatment period and signs of hypoglycemia three days after the starting treatment. In the experiments by Kronfeld et al. (1963) with higher doses of insulin, numerous cows became recumbent and depressed during treatment with successive doses of insulin, and during these severe hypoglycemic episodes, the authors report that the cows did not eat. Hypoglycemia, as opposed to insulin itself, was likely the cause of reduced milk yield, as restoration of normal plasma glucose concentrations by exogenous glucose administration in cows treated with insulin also restored milk production (Kronfeld et al., 1963). Maintenance of milk yield is commonly observed during hyperinsulinemic-euglycemic clamps (McGuire et al., 1995b; Mackle et al., 1999), which further supports the concept that hypoglycemia is the cause of reduced milk yield when insulin is administered at large doses.

There was a tendency for increased milk fat content and yield with slow-release insulin treatment ($P = 0.12$ and $P = 0.13$ for milk fat content and yield, respectively,

Table 4-2). The tendency for a treatment difference appears to be driven largely by the 10% increase in fat content and yield in cows treated with L (contrast C vs. L, $P < 0.05$), as there was no effect of H on milk fat content ($P = 0.36$) or yield ($P = 0.44$). The increase in milk fat production for cows treated with L was unexpected as studies with hyperinsulinemic-euglycemic clamps have reported no change (McGuire et al., 1995b) or a decrease in milk fat content (Griinari et al., 1997b; Mackle et al., 1999; Bequette et al., 2001; Corl et al., 2006). Similarly, in study by Leonard and Block (1997), milk fat content and yield were reduced by 15 and 21%, respectively, in cows treated with both insulin and glucose compared to saline-infused control cows. During close arterial infusion of insulin to the mammary gland, a non-significant increase in milk fat yield was observed (Metcalf et al., 1991). In the present study, it is unclear why cows treated with L had higher milk fat yield. Though insulin does not regulate glucose uptake by the mammary gland, mammary epithelial cells exhibit responsiveness to insulin. The discrepancy between the lack of response of cows treated with H compared to the response by cows treated with L may be related to the altered amino acid structure of H relative to L (Owens and Bolli, 2008). As L is an analogue of insulin, the amino acid substitutions to the molecule may alter its insulin receptor affinity and residence time or make the analogue have greater affinity for receptors of the insulin-like growth factor family (Kurtzhals et al., 2000; Staiger et al., 2007; Shukla et al., 2009).

Milk protein content and yield were both affected by administration of slow-release insulin (Table 4-2). A treatment by day interaction was observed for milk protein yield such that responses to treatment increased over the 10-d treatment period (Figure 4-1). Though milk protein content for cows treated with H was 6.7% higher than control cows, milk protein yield did not differ between those two treatments. On the other hand, milk protein content and yield were 9.6 and 5.5% higher, respectively, for cows treated with L when compared to control cows. A possible explanation for the greater response in protein yield by cows treated with L may be related to the overall

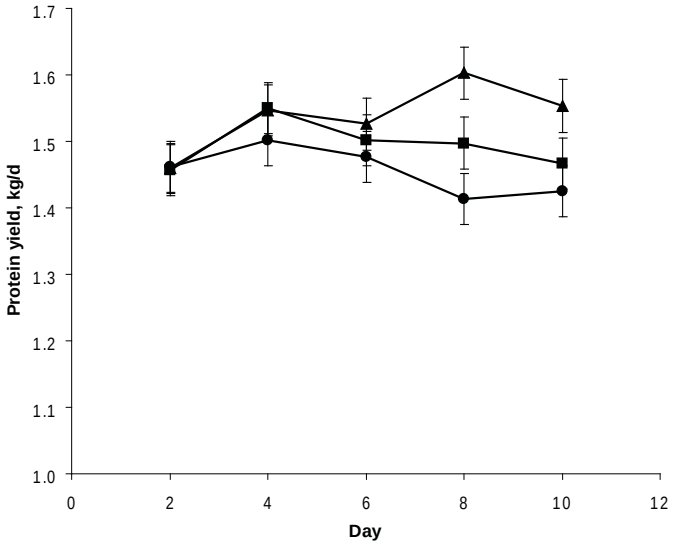

Figure 4-1. *Milk protein yield, kg/d, for control cows (–●–), cows treated twice daily with 0.2 IU/kg BW of Humulin-N (–■–), or cows treated twice daily with 0.2 IU/kg BW of insulin glargine (–▲–) treatments. Treatments were given by subcutaneous injection at 1000 h and 2200 h for 10 d. Values shown are the least square means and their associated standard errors. Probabilities of significance for effects of treatment, day, and treatment x day interaction were P = 0.084, P = 0.001, and P = 0.076, respectively.*

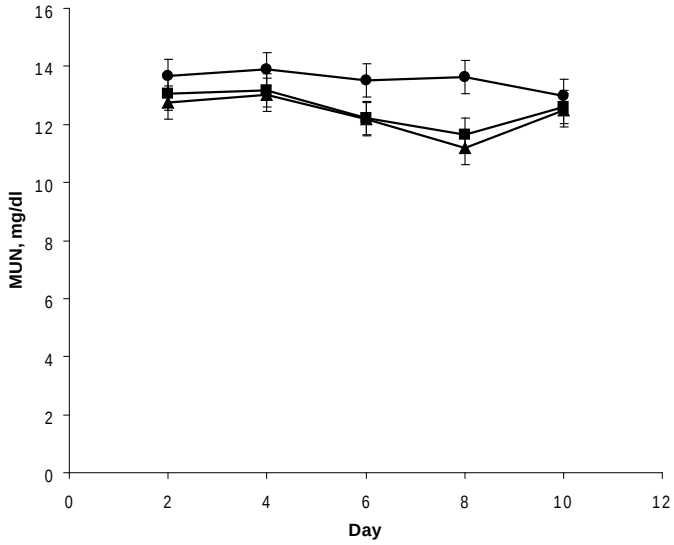

Figure 4-2. *Milk urea nitrogen, mg/dl, for control cows (–●–), cows treated twice daily with 0.2 IU/kg BW of Humulin-N (–■–), or cows treated twice daily with 0.2 IU/kg BW of insulin glargine (–▲–) treatments. Treatments were given by subcutaneous injection at 1000 h and 2200 h for 10 d. Values shown are the least square means and their associated standard errors. Probabilities of significance for effects of treatment, day, and treatment x day interaction were P = 0.012, P < 0.001, and P = 0.076, respectively.*

amount of insulin-activity in these cows. Based on plasma glucose concentrations, it would appear that cows treated with L had higher insulin activity during the 10-d treatment period as evidenced by lower plasma glucose than cows treated with H. Milk urea nitrogen concentrations were decreased by administration of both forms of slow-release insulin and least square means for MUN were 13.5, 12.5, and 12.3 ± 0.52 mg/dl for C, H, and L treatments, respectively. A treatment by day interaction was observed for MUN ($P = 0.076$) and this interaction is shown in Figure 4-2. Lowered MUN concentrations suggest greater efficiency of use of N in cows treated with slow-release insulin. Using the means for protein yield and DMI, and estimated MP supply, the efficiency of MP use for milk protein was 0.446, 0.459, and 0.463 g of true milk protein per g of MP for the control, H, and L treatments, respectively. These values are lower than those reported by Metcalf et al., (2008) and the values reported by Metcalf et al., include a correction for MP maintenance requirements. If the present data are adjusted for the amount of MP used for maintenance, the efficiency of MP use for milk protein becomes 0.626, 0.644, and 0.649 g of true milk protein per g of MP for the C, H, and L treatments, respectively, which are much closer to the efficiency values reported by Metcalf and coworkers (2008) of 0.62 to 0.64.

In this experiment, lactose content and yield were reduced by treatment ($P = 0.001$ and $P = 0.07$, for content and yield, respectively) with both slow-release insulins. For cows treated with H, lactose content was reduced by 1.7% ($P = 0.02$), while yield had a tendency to be reduced by 3.4% ($P = 0.13$) when compared to control cows. For cows treated with L, the reduction in lactose content was 2.9% ($P < 0.001$) and the reduction in yield was 5.6% ($P = 0.02$) versus control cows. As lactose is considered to be the major osmoregulator of milk and thus dictates milk volume, these significant reductions in milk lactose yield may be the explanatory variable behind the non-significant, but numerical declines in milk yield for cows treated with both slow-release insulin types. In the series of experiments by Schmidt (1966), insulin injections

Table 4-3. *Effect of twice-daily subcutaneous injections of two types of slow-release insulin on milk nitrogen fractions and protein composition for composite milk samples collected on day 10 of treatment*

	Treatment[1]				*P*-value		
Variable	C	H	L	SEM[2]	Trt	C vs. H	C vs. L
CP,[3] %	3.17	3.36	3.41	0.146	0.006	0.013	0.003
CP, kg/d	1.53	1.57	1.66	0.116	0.070	0.455	0.027
NCN,[4] %	0.69	0.72	0.75	0.031	0.006	0.058	0.002
NCN, kg/d	0.33	0.34	0.36	0.024	0.111	0.480	0.044
NPN, [4] %	0.18	0.17	0.18	0.01	0.761	0.607	0.855
NPN, kg/d	0.09	0.08	0.09	0.01	0.591	0.511	0.759
True Protein,[5] %	3.00	3.19	3.22	0.14	0.003	0.007	0.002
True Protein, kg/d	1.44	1.49	1.58	0.11	0.057	0.397	0.021
Casein, [6] %	2.49	2.63	2.65	0.124	0.019	0.020	0.012
Casein, kg/d	1.20	1.23	1.30	0.094	0.069	0.446	0.026
Casein, % of True Protein	82.8	82.6	82.5	0.64	0.696	0.462	0.465

[1] Treatments: C = control, H = 0.2 IU/kg BW Humulin-N, L = 0.2 IU/kg BW insulin glargine given twice daily via subcutaneous injection at 1000 h and 2200 h.
[2] SEM = standard error of the mean.
[3] Crude protein is equal to total nitrogen (TN) x 6.38.
[4] Both NPN and NCN are multiplied by 6.38 to allow comparison with other protein fractions.
[5] True protein is calculated as (TN - NPN) x 6.38.
[6] Casein protein is calculated as (TN - NCN) x 6.38.

also resulted in reduced lactose content and yield. Similarly, cows given insulin and glucose (Leonard and Block, 1997) in early lactation also had reduced milk lactose yield. Rook et al. (1965) observed that when plasma glucose fell below 40 mg/dl, milk yield and lactose yield dramatically decreased, suggesting a baseline need for plasma glucose in total lactose yield. Though glucose is the main substrate for lactose synthesis, it does not appear to be the only driver for lactose synthesis as studies using phlorizin to increase glucose disposal in the urine have shown no change in lactose synthesis in lactating dairy cows (Amaral-Phillips et al., 1993; Bradford and Allen, 2007). However, plasma glucose concentrations in these studies were only reduced nominally despite large quantities of urinary glucose disposal. Work by Bradford and Allen (2005) in late lactation cows suggests that cows at this stage of lactation have sufficient gluconeogenic capacity in the liver to overcome the phlorizin-induced glucose deficit.

Cows treated with L had 4% higher total solids content in their milk compared to control cows ($P = 0.024$; Table 4-2). There was no difference between cows treated with H and control animals ($P > 0.05$). However, with the non-significant reduction in milk yield for cows treated with L, there was no difference in total solids yield by treatment and this was also observed with no effects of treatment on energy-corrected milk yield (Table 4-2). Somatic cell counts in milk were not affected by treatment ($P > 0.05$).

Milk Nitrogen Fractions and Protein Composition

Milk nitrogen fractions and protein composition are presented in Table 4-3. Crude protein, as equal to the total N content of the milk multiplied by 6.38, was increased by treatment with slow-release insulin. Crude protein content was 6% higher for cows treated with H and 7.6% higher for cows treated with L compared to control cows. Crude protein yield was 8.5% higher for cows treated with L compared to control cows, but crude protein yield for cows treated with H did not differ from controls. True protein, as reported in Table 4-3, was calculated by subtracting NPN from TN and multiplying by 6.38. True protein content was 6.3 and 7.3% higher for cows treated with H and L, respectively, and the TP yield was 9.7% higher for cows treated with L. The mean TP content for each treatment, as determined by Kjeldahl analysis, were as follows: 3.00, 3.19, and 3.22 ± 0.14 for C, H, and L, respectively.

The TP content and yields determined by this method correlate well with the TP values reported in Table 4-2, which were determined by Dairy One Cooperative, Inc. (Ithaca, NY) by mid-infrared spectroscopy. Slight differences could be expected between these two TP values as the values in Table 4-2 from Dairy One represent samples taken twice per day on every other day of the treatment period, while the values reported in Table 4-3 are the results from a composite sample of the 2 milking times collected only on d 10 of the study.

Table 4-4. *Effect of twice-daily subcutaneous injections of two types of slow-release insulin on plasma glucose, non-esterified fatty acids (NEFA), urea nitrogen (PUN), and insulin concentrations*

	Treatment[1]				*P*-value								
Variable	C	H	L	SEM[2]	Trt	Day	Trt x Day	Time[3]	Trt x Time	Day x Time	Trt x Day x Time	C vs. H	C vs. L
Glucose, mg/dl	56.8	52.0	48.1	0.99	<0.001	0.170	0.707	0.003	0.001	0.075	0.175	<0.001	<0.001
NEFA, µeq/L	166	197	181	9.1	0.046	0.008	0.881	<0.001	0.858	<0.001	0.384	0.013	0.230
PUN, mg/dl	11.4	11.2	10.5	0.49	0.009	<0.001	0.807	<0.001	0.063	<0.001	0.636	0.572	0.004
Insulin, ng/ml	0.830	0.971	0.952	0.0398	0.020	0.158	0.866	<0.001	0.077	0.190	0.566	0.009	0.032

[1] Treatments: C = control, H = 0.2 IU/kg BW Humulin-N, L = 0.2 IU/kg BW insulin glargine given twice daily via subcutaneous injection at 1000 h and 2200 h.
[2] SEM = standard error of the mean.
[3] Time represents the difference in plasma samples taken immediately before treatment administration at 1000 h and 6 h later at 1600 h

Casein content of the milk was increased by treatment with slow-release insulin and was about 6% higher for cows treated with either H or L compared to controls. Casein yield was 8.3% higher for cows treated with L ($P = 0.01$) when compared to controls, but yield from cows treated with H was not different from that of the control cows ($P = 0.45$). Though there was an increase in the casein content of the milk for cows treated with both forms of slow-release insulin, the composition of the major milk proteins was not affected by treatment ($P = 0.70$) as the casein percentage of true protein was maintained near 82.5% for all treatments. Under hyperinsulinemic-euglycemic clamp conditions, other studies have also observed no change in the composition of the major milk proteins (Griinari et al., 1997a; Mackle et al., 1999).

Noncasein nitrogen content increased with treatment of both H and L, and the response was greater for L (Table 4-3). Additionally, the yield of NCN tended to be higher by treatment ($P = 0.111$). Nonprotein nitrogen content and yield were not affected by treatment with either form of slow-release insulin ($P > 0.10$). Mackle et al. (1999) reported similar observations for NCN and NPN under hyperinsulinemic-euglycemic clamp conditions.

Plasma Hormones and Metabolites

Results for plasma glucose, NEFA, PUN, and insulin concentrations in response to treatment with slow-release insulins are presented in Table 4-4. As expected, plasma glucose concentrations were lower for cows treated with either H or L when compared to control cows. Cows treated with H had 8.5% lower plasma glucose, while cows treated with L had 15.3% lower plasma glucose concentrations. The mean plasma glucose concentrations were 56.8, 52.0, and 48.1 ± 0.99 mg/dl for C, H, and L, respectively. These results are similar to the decreases observed in our dose response study (Winkelman et al., 2010), though those results represent only a

24-h sampling period of hourly samples. As with the aforementioned study, there were no visually apparent signs of hypoglycemia during the experiment, which suggested that cows at this stage of lactation had sufficient gluconeogenic capacity to adapt their glucose homeostasis to the administration of slow-release insulin. However, it would be expected that at cows did experience transient states of hypoglycemia throughout the day, based on the 95% confidence intervals around the mean plasma glucose values for the 24-h dose response study, as discussed in Chapter 3.

Though there was an overall effect of treatment on plasma NEFA ($P = 0.008$), this difference was driven by 19% higher NEFA in cows treated with H (compared to C; $P = 0.013$), as NEFA were not different between control cows and those treated with L ($P = 0.23$). Although NEFA were higher for cows treated with H, the concentrations observed are within normal physiological range for cows at this stage of lactation. Increased plasma NEFA concentrations were not expected for either of the slow-release insulin treatments because insulin suppresses lipolysis and promotes lipogenesis in adipose tissue (Brockman, 1978). Recall that milk fat content and yield were higher for cows treated with L, but not statistically different for H. This observation, in combination with no change in plasma NEFA concentration for cows treated with L, suggests that de novo synthesis of milk fatty acids was stimulated by treatment with L, but not H. If this theory is true, the proportion of medium and short-chain fatty acids in milk would be higher for cows treated with L (Palmquist et al., 1993) and may account for the difference in milk fat output observed here.

Plasma urea nitrogen concentrations were affected by treatment ($P < 0.01$), and this observation was largely driven by treatment with L as PUN concentrations were 8% lower in cows treated with L as compared to control cows. Plasma urea nitrogen concentrations over the 10-d treatment period were 11.4, 11.2, and 10.5 ± 0.49 mg/dl for C, H, and L treatments, respectively. Under the experimental context of the hyperinsulinemic-euglycemic clamp when cows produced 15% more milk protein,

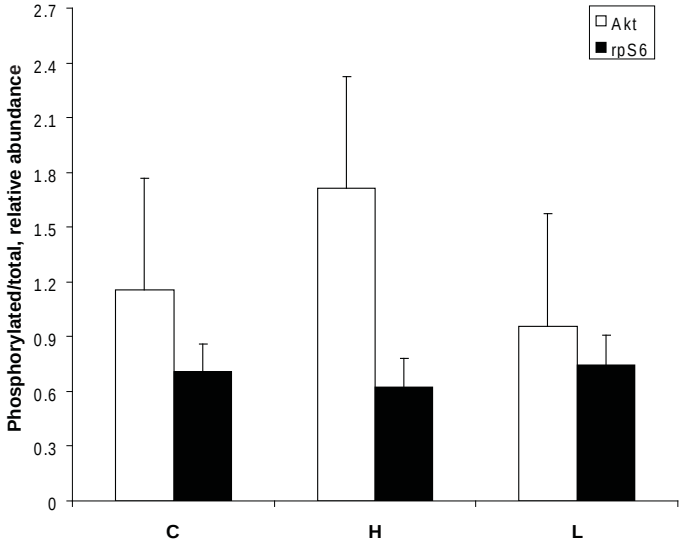

Figure 4-3. *Effect of twice-daily subcutaneous injections of two different slow-release insulins on signaling molecules in the mammary gland. Treatments consisted of control (C), and treatment with either Humulin-N (H) or insulin glargine (L) at a dosage rate of 0.2 IU/kg BW twice daily at 12-h intervals for a total daily dose of 0.4 IU/kg BW. Bars represent least-square means (± SEM) of phosphorylated to total relative abundance ratios of arbitrary density units. Treatment affected the ratio of phosphorylated to total Akt (P = 0.05) and was highest for H. There was no effect of treatment on the ratio of phosphorylated to total ribosomal protein S6 (rpS6; P = 0.60).*

PUN concentrations were 31.5% lower than control animals (Mackle et al., 1999). As blood urea rapidly equilibrates with milk urea (Gustafsson and Palmquist, 1993), the reduction in PUN observed here corresponds with the nearly 9% reduction in MUN (Table 4-2), improved milk protein yield, and incremental improvement in the conversion of MP into milk protein.

Plasma insulin concentrations as measured by RIA were higher for cows treated with either H or L. Due to challenges in measuring L and its metabolites in plasma (Winkelman et al., 2010 and discussed in Chapter 3), the values for cows treated with L may not be completely indicative of overall insulin activity within these cows. Regardless, as plasma glucose concentrations were lower in all cows treated with either form of slow-release insulin, we can reasonably state that insulin-like activity was elevated in cows treated with H and L.

Cell Signaling

The ratio of phosphorylated to total abundance of Akt and rpS6 are shown in Figure 4-3. Treatment affected the phosphorylation ratio of Akt ($P = 0.05$), but not rpS6 ($P = 0.60$). Cows treated with H had the highest phosphorylation state of Akt. Because no changes in the signaling molecule downstream of mTOR (rpS6) were observed in the lysates, no further analysis was completed on the mammary protein lysates. The actual western blot images used in densitometry analysis can be found in the Appendix.

There are a few reasons why no detectable or more significant differences in these signaling components were observed in the present study. First, the amount of time (12 to 15 h) that passed between the last treatment dose and the time of biopsy may have been longer than optimal to evaluate changes in phosphorylation state of these proteins. Changes in phosphorylation state occur rapidly, within minutes after stimulation, (Bolster et al., 2004; Burgos and Cant, 2010), thus the biopsies taken in this study would have missed the acute changes in phosphorylation status of these proteins. However, as both H and L have extended duration of action, we thought the changes in phosphorylation of these proteins may have been observable at the time of the biopsy. In addition to acute changes to stimulate protein synthesis, insulin exerts a longer term effect by increasing the capacity for protein synthesis by increasing the number of ribosomes (Bolster et al., 2004; Proud, 2006; Mahoney et al., 2009). By the time the mammary biopsies were taken on d 11, it would be possible that changes in phosphorylation state of Akt and rpS6 by 12 to 15 h post-insulin treatment would be masked by a greater overall capacity of the tissue to synthesize proteins such that the phosphorylation ratios would not be different.

Additionally, biopsies were taken relatively soon after milk removal (within 3 h post milking). The respiratory quotient of the mammary gland is highest during the first 6 h following milk removal (Thivierge et al., 2002a), suggesting high conversion of nutrients rich in oxygen to products with relatively low oxygen content, such as lipids and

proteins. The mammary gland is a tissue of high metabolic activity and protein synthesis by the mammary gland accounts for more than 40% of whole-body protein synthesis (Thivierge et al., 2002b). With high rates of metabolism and synthesis occurring during this period following milking, it would be possible that the protein synthetic machinery in the mammary gland was highly active for all the cows at this time and not just the cows treated with slow-release insulin. Given these possible explanations for the lack of significant changes in the signal transduction proteins evaluated here, it would have been ideal to alter the timing of the mammary biopsy to be closer to the time of treatment. Additionally, it would have been ideal to have a biopsy collected prior to treatments commenced as a covariate point of adjustment in analysis.

CONCLUSIONS

Insulin appears to play a role in milk protein synthesis based on the results reported here with the use of slow-release insulin products and confirms the conclusions drawn from studies using hyperinsulinemic-euglycemic clamps in lactating cows (Griinari et al., 1997a; Mackle et al., 1999). Using the slow-release insulins described here provide an alternative method to study the effects of insulin without large decreases in glycemic state or severe hypoglycemia. Further work needs to be completed to explain the improvement in milk protein and fat yield reported here, but we hypothesize that signaling through the mammalian target of rapamycin pathway is altered under elevated insulin activity (Proud, 2006).

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CHAPTER FIVE: EFFECTS OF INSULIN GLARGINE ON MAMMARY AMINO ACID UTILIZATION BY LACTATING DAIRY COWS

ABSTRACT

Three multiparous lactating Holstein dairy cows received twice-daily subcutaneous injections of insulin glargine (0.15 IU/kg BW per injection, total daily dose 0.3 IU/kg BW) or sterile water at 12-h intervals for 4 d in a complete crossover design with two periods. Cows were randomized to one of two sequences, either insulin treatment followed by control, or control followed by insulin treatment. On d 4 of the treatment period, simultaneous blood samples were collected from catheters inserted in the intercostal artery and subcutaneous abdominal vein at hourly intervals for 12-h. Treatment with insulin glargine decreased plasma glucose concentrations in both the arterial ($P < 0.001$) and venous blood supplies ($P < 0.001$). Mean arterial glucose concentrations were 63.1 and 56.9 mg/dl for the control and insulin glargine treatment periods, respectively. Mammary blood flow was nonsignificantly decreased during treatment with insulin glargine. Mammary uptake of both essential ($P = 0.059$) and nonessential ($P = 0.086$) amino acids were reduced during insulin glargine treatment. No effects of treatment were observed for dry matter intake or most milk production variables. Milk yield tended to be lower ($P = 0.15$) during the insulin glargine treatment, but subclinical mastitis in one cow during the insulin glargine treatment period may have contributed to this response. Milk lactose content ($P = 0.094$) and yield ($P = 0.091$) decreased with treatment of insulin glargine. Casein expressed as a percentage of true protein did not differ between treatments ($P = 0.34$), but noncasein N content was increased by treatment with insulin glargine ($P = 0.006$). Overall these results suggest that insulin glargine altered efficiency of use of amino acids by the mammary gland as evidenced by reduced uptake of both essential and nonessential amino acids but no change in milk protein output.

INTRODUCTION

Milk protein continues to be the most valuable milk component under the multiple component pricing system in the dairy industry. Altering diets to improve protein yield have had only marginal success (Sutton, 1989), particularly in well-fed dairy cows. The use of hyperinsulinemic-euglycemic clamps in lactating dairy cows (McGuire et al., 1995; Griinari et al., 1997; Mackle et al., 1999b) has been shown to increase milk protein content and yield by up to 28%, suggesting that milk protein yield under current management conditions is not maximized. Additionally, the efficiency of converting dietary nitrogen (N) into milk protein output is relatively poor in the lactating ruminant, between 25 to 30 percent (Bequette et al., 1998). The conversion of metabolizable protein to milk true protein approximates 0.62 to 0.64 g of milk true protein per g of metabolizable protein (Metcalf et al., 2008). While conversion of MP to productive N is higher than the conversion of dietary N to productive N, there is still opportunity to improve N utilization within the ruminant. Improving N efficiency within the cow will help the industry to avoid costly N loss to the environment, as low N efficiency of use leads to greater losses of N in urine (Broderick, 2003).

During hyperinsulinemic-euglyemic clamp studies in lactating ruminants, arterial concentrations of the branched-chain amino acids are decreased (Griinari et al., 1997; Mackle et al., 1999b; Mackle et al., 2000), but the extraction efficiencies of these amino acids are increased (Mackle et al., 2000). While these observations are attributed to the effects of elevated circulating insulin concentrations, the confounding effects of large amounts of glucose, and thus energy (Leonard and Block, 1997), may also be playing a role in these changes in amino acid supply and uptake by the lactating ruminant mammary gland. Close arterial glucose infusion at the mammary gland decreases blood flow to the gland (Cant et al., 2002) and therefore it is reasonable to assume that glucose infusion during hyperinsulinemic-euglycemic clamps may also exerts changes in mammary metabolism and nutrient use by the mammary gland.

The goal of the present research was to further define the role of insulin in changes in mammary usage of amino acids through use of a form of slow-release insulin to modestly increase insulin activity in lactating dairy cows without large changes in glycemia. In the present experiment, the slow-release insulin analogue, insulin glargine (Lantus, Sanofi-Aventis, Bridgewater, NJ) was used to determine its effects on mammary uptake and utilization of amino acids in well-fed lactating dairy cows.

MATERIALS AND METHODS

Animals, Experimental Design, and Treatments

The Cornell University Institutional Animal Care and Use Committee approved all procedures involving the dairy cows used in the experiment described below. Three multiparous Holstein cows (101 DIM ± 22 d) with indwelling intercostal arterial catheters were used in this experiment. The cows with the indwelling intercostal arterial catheters were carried over from a previous experiment (Hofherr et al., 2010) and the catheter patency was maintained in between the end of that aforementioned experiment until the end of present study. Experimental design consisted of a complete crossover of treatments with a washout period occurring in between periods. Treatment periods lasted 4 d, and the washout period was 2 d.

Cows were offered a common total mixed ration (TMR; Table 5-1) daily at 0900 h and orts were removed before feeding. The diet was formulated to meet NRC recommendations for nutrients (National Research Council, 2001). Amounts of feed offered and refused were measured on a daily basis, and samples of the TMR were collected daily and dried at 55°C until static weight was reached for measurement of DM content. Daily DMI were calculated for each cow using the respective amounts of feed consumed and the appropriate daily DM content of the TMR. Samples of the TMR were composited within period and sent to Cumberland Valley Analytical

Table 5-1. *Dietary ingredients and nutrient composition of the basal diet (DM basis)*

Ingredient, %	Content
Corn silage	45.1
Alfalfa silage	14.5
Concentrate mix[1]	18.1
Concentrate mix[2]	7.3
Corn meal	7.2
Corn distillers grain	7.2
Energy Booster[3]	0.6
Energy and nutrients[4]	
NE_L, Mcal/kg	1.72
CP,	17.3
Soluble Protein, % CP	45.9
RDP, % CP	73.0
ADF	20.1
NDF	34.2
NFC	38.9
Ash	6.5
Calcium	0.59
Phosphorus	0.40
Magnesium	0.30
Potassium	1.33
Sodium	0.5
Iron, ppm	223
Manganese, ppm	789
Zinc, ppm	101
Copper, ppm	18

[1] The concentrate mix was comprised of (DM basis): 27.1% corn germ meal; 16.5% citrus pulp, 11.5% wheat midds, 11.6% bakery product, 5.3% cereal tailings, 5.2% blood meal, 4.0% molasses, 3.2% soybean meal, 3.4% bypass fat, 2.9% calcium carbonate, 2.8% sodium bicarbonate, 1.8% salt, 1.6% urea, 0.9% Optigen II (Alltech, Lexington, KY), 0.8% tallow, 0.6% magnesium oxide, 0.3% Alimet (88% aqueous solution of dl, 2-hydroxy-4-(methylthio) butanoic acid, Novus International, Inc., St. Louis, MO), 0.2% selenium 0.06%, 0.1% trace minerals, 0.1% calcium salt of dl-methionine, 0.1% Agrado (Novus International, Inc., St. Louis, MO), 0.1% vitamin A/D/E.

[2] The concentrate mix was comprised of (DM basis): 50.7% corn germ meal; 19.2% citrus pulp, 4.9% blood meal, 4.8% bakery product, 4.0% molasses, 4.2% calcium carbonate, 3.1% sodium bicarbonate, 2.2% salt, 2.0% dextrose, 1.9% urea, 1.4% Optigen II (Alltech, Lexington, KY), 0.6% magnesium oxide, 0.3% Alimet (88% aqueous solution of dl, 2-hydroxy-4-(methylthio) butanoic acid, Novus International, Inc., St. Louis, MO), 0.3% selenium 0.06%, 0.2% trace minerals, 0.2% calcium salt of dl-methionine, 0.1% Agrado (Novus International, Inc., St. Louis, MO), 0.1% vitamin A/D/E.

[3] Prilled saturated FFA, MS Specialty Nutrition, Dundee, IL.

[4] Values represent average of 3 weekly TMR samples composited into one sample for analysis.

Services (Hagerstown, MD) for analysis using wet chemistry techniques for CP (AOAC, 2000), acid detergent insoluble CP [CP determined using AOAC (2000) after acid detergent extraction (AOAC, 2000)], neutral detergent insoluble CP [AOAC (2000) method for CP after neutral detergent extraction (Van Soest et al., 1991)], soluble CP, (Krishnamoorthy et al., 1982), ADF (AOAC, 2000), NDF (AOAC, 2000), lignin (Goering and Van Soest, 1970), starch (Hall, 2009), sugar (Dubois et al., 1956), ether extract (AOAC, 2000), and ash (AOAC, 2000). Chemical composition of the basal TMR based on the composition of the components is reported in Table 5-1. During the experiment, free choice water was available at all times.

During the first period, two cows received subcutaneous insulin glargine (L) treatments of 0.15 IU/kg BW twice daily at 0900 and 2100 h, for a total daily dose of 0.3 IU/kg BW. The third cow served as a control in the first period and received a 2-ml injection of sterile water. Treatments were reversed in the second period. On d 4 of each period, simultaneous blood samples were collected from intercostal arterial and abdominal subcutaneous mammary vein catheters at hourly intervals for 12 h beginning at 0900 h and transferred into heparinzed glass tubes. Plasma was harvested by centrifugation (3,000 x *g* for 20 min at 4ºC) and plasma was harvested into three aliquots, quickly frozen with liquid N_2, and then stored at -20ºC. An additional aliquot of plasma (0.65 ml) was added to ice-cold sulfosalicylic acid (5%) containing norleucine (2.5 µM) as an internal standard for amino acid analysis. The mixture of plasma and sulfosalicylic acid was vortexed extensively and kept on ice at 40ºC overnight. The following day, after vortexing again, the plasma and sulfosalicylic acid mixture was centrifuged for 10 min at 4°C (15,800 x *g*). For each cow, the deproteinized plasma from the 12 hourly samples were composited at 2-h intervals to give six pairs of arterial and venous samples for each day within each period. Deproteinized plasma samples were frozen at -80ºC until analysis for AA by HPLC (Mackle et al., 1999a).

To calculate uptake of amino acids and blood constituents by the mammary gland from AV differences, mammary blood flow was estimated using the Fick principle according to the method described by Cant et al (1993), which assumes that tissue uptake = AV difference x blood flow. Phenylalanine and tyrosine were used as the indicator compounds as both are transferred stoichiometrically from blood into milk protein (Mepham, 1982). Blood proteins were assumed to be 3.5% of the total milk protein and the following equation was used to calculate blood flow:

$$MBF = (FY_B \times 0.965)/FY_{A-V}$$

where:

FY_B = Phe + Tyr output in milk protein (moles per hour), and

FY_{A-V} = Phe + Tyr AV differences (moles per liter).

Cows were milked three times daily at 0700, 1500, and 2300 h and individual milk weights were recorded. On d 4 of each treatment period, milk samples were obtained at each milking and stored at 4°C with a preservative (2-bromo-2-nitro-1, 3 propanediol) until analyzed (Dairy One Cooperative Inc., Ithaca, NY) fat, protein, lactose using midinfrared spectroscopy (AOAC, 2000; method 972.160), and SCC by an optical fluorescent method (AOAC, 2000; method 978.26). A second aliquot of samples was collected and samples were composited by day in proportion to individual milk weights, representing d 3 and 4 of the treatment period. These composite samples were analyzed for milk N fractions. Casein was determined by the indirect method (Lynch et al., 1998) such that casein was precipitated at pH 4.6 in acetic acid and sodium acetate, and noncasein nitrogen (NCN) in the filtrate was measured by Kjeldahl N analysis. Total nitrogen (TN) of the sample was also measured by Kjeldahl analysis (Barbano et al., 1990). The amount of casein (N x 6.38) in the sample was calculated as the difference between the amount of TN and NCN. To determine NPN, proteins were precipitated and filtered from the sample by addition of 12% (wt.:vol.) trichloroacetic acid (Barbano et al., 1991). The N content of the filtrate, as measured by Kjeldahl N

analysis, is the NPN (N x 6.38) fraction. True protein can be calculated by difference taking the difference between TN and NPN (N x 6.38).

Plasma Hormone and Metabolite Analysis

Plasma concentrations of glucose were determined by enzymatic analysis (glucose oxidase) using a commercially-available reagents (#P7119 PGO Enzyme Preparation, #F5803 3,3'Dimethoxy benzidine dihydrochloride; Sigma Aldrich, St. Louis, MO). Intra-assay and interassay coefficients of variation (CV) for glucose analysis were 2.6 and 3.3%, respectively. Plasma urea nitrogen was measured by the hypochlorite-phenol nitroprusside method using a commercially available enzymes and reagents (#U3383 Urease Buffer Reagent, #P6994 Phenol Nitroprusside solution, #A1727 Alkaline Hypochlorite solution; Sigma Aldrich, St. Louis, MO). Intra-assay and interassay coefficients of variation for PUN analysis were less than 4 and 7%, respectively. Spectrophotometric readings were made using a $Versa_{max}$ tunable microplate reader (Molecular Devices, Sunnyvale, CA). Plasma insulin concentration was determined by a commercially available radioimmunoassay kit which has 90% cross-reactivity with native bovine insulin (#PI-12K Porcine Insulin Radioimmunoassay, LINCO Research, St. Charles, MO). Intra-assay and interassay CV for the RIA insulin analysis were both 10%.

Statistical Analysis

Data were subjected to analysis of variance using the MIXED procedure of SAS (SAS Institute, 2001). Data presented here represent data collected on d 4 of the treatment period. The model included the fixed effects of treatment, sequence of treatments, and the interaction of treatment x sequence. Significance was declared at $P < 0.10$ and trends for $0.10 < P < 0.15$. Least squares means are presented throughout.

RESULTS AND DISCUSSION

Intake, Milk Production, and Milk Components

The ingredient and nutrient composition of the basal diet is presented in Table 5-1. Dry matter intake averaged 25.7 kg/d and did not differ between treatments (Table 5-2). Insulin, when given alone or in combination with glucose, has been reported to have mixed effects on intake in ruminants. Cows given regular insulin at a dose of 0.3 U/kg BW (Schmidt, 1966) after each milking had decreased milk production during the treatment period and signs of hypoglycemia three days after the starting treatment. In early lactation, injection of slow release insulin tended exert a quadratic effect on in DMI, with the highest DMI occurring at a insulin dose of 0.14 IU/kg BW per day (Hayirli et al., 2002). In sheep, insulin effects on DMI were also variable (Deetz et al., 1980), sometimes causing a decrease in intake while at other times having no effect on intake depending on dose or route of administration.

The effects of treatment on milk yield and composition are reported in Table 5-2. Milk yield tended to be 1.5 kg lower during the L treatment ($P = 0.15$). In a 10-d production study using L at a higher dose (0.2 IU/kg BW, twice daily) and discussed in Chapter 4, there was no effect of L on milk yield. Milk protein and fat contents and yields were not affected by treatment. Given the limited number of cows in the present study, differences in production variables were not expected but are reported here for reference in later discussion regarding amino acid uptake and utilization. While some studies have shown positive effects of insulin (through hyperinsulinemic-euglycemic clamps) on milk protein yield (McGuire et al., 1995; Griinari et al., 1997; Mackle et al., 1999b), others have shown no change in milk protein yield (Metcalf et al., 1991; Molento et al., 2002). The differences in milk protein yield in other studies may have been driven by a greater elevation in circulating insulin concentrations. In the present study and those by Metcalf et al. (1991) and Molento et al (2002), insulin concentrations were much lower than those from the hyperinsulinemic-euglycemic clamp studies of

Table 5-2. *Production responses to twice-daily subcutaneous injections of insulin glargine*

	Treatment[1]			*P*-value
Variable	Control	Insulin glargine	SEM	Trt
DMI, kg/d	25.30	26.13	2.12	0.33
Milk, kg/d	47.16	45.64	4.34	0.15
Fat, %	3.64	3.76	0.36	0.31
Fat yield, kg/d	1.73	1.73	0.33	0.88
Protein, %	2.94	3.11	0.19	0.39
Protein yield, kg/d	1.39	1.42	0.22	0.74
Lactose, %	4.94	4.83	0.03	0.094
Lactose yield, kg/d	2.33	2.21	0.21	0.091
Total solids, %	12.42	12.58	0.57	0.18
Total solids yield, kg/d	5.88	5.76	0.80	0.37
SCC, cells/ml (x 1,000)	600	911	1160	0.67

[1]During control treatment, cows received twice-daily subcutaneous injections of 2 ml of sterile water. During insulin glargine treatment, cows received twice-daily subcutaneous injections of insulin glargine (0.15 IU/kg BW), for a total daily dose of 0.30 IU/kg.

McGuire et al. (1995), Griinari et al. (1997) and Mackle et al. (1999b; 2000) where insulin concentrations were 4 to 5-fold higher than baseline concentrations.

Lactose content and yield were both reduced by 2.1 and 5.1%, respectively, by twice-daily treatment with L. Studies using hyperinsulinemic-euglycemic clamps or insulin plus glucose infusions have also observed decreases in milk lactose concentrations and yields (Schmidt, 1966; Leonard and Block, 1997), whereas glucose infusions increase milk lactose concentrations (e.g. Leonard and Block, 1997; Cant et al., 2002). Though glucose is the main substrate for lactose synthesis, it does not appear to be the only driver for lactose synthesis as studies using phlorizin to increase glucose disposal in the urine have shown no change in lactose synthesis in lactating dairy cows (Amaral-Phillips et al., 1993; Bradford and Allen, 2007), though plasma glucose concentrations in these studies were only reduced nominally despite large quantities of urinary glucose disposal. Work by Bradford and Allen (2005) in late lactation cows suggests that cows at this stage of lactation have sufficient gluconeogenic capacity in the liver to overcome the phlorizin-induced glucose deficit.

Though SCC was not affected by treatment ($P = 0.67$), the average SCC was high and there was large variation in SCC due to the high somatic cell counts of one animal. During the first period, one cow on the L treatment unexpectedly had a high SCC and this carried over to the second period, though the SCC was not as high as in the first period for this animal. The effects of elevated SCC are relevant to the discussion of milk protein composition and interpretation of the protein composition results. Elevated SCC and subclinical mastitis are associated with reduced milk yield (Hagnestam-Nielsen et al., 2009) and may help explain the trend for reduced milk yield observed in this study. In another study where insulin was given without glucose, there was no change in milk production (Metcalf et al., 1991), suggesting insulin can be given by itself without severe hypoglycemia and losses in production.

Milk Nitrogen Fractions and Protein Composition

Results for milk N composition and fractions are reported in Table 5-3. The TP concentrations as determined by Kjeldahl analysis were similar to the results obtained from analysis by Dairy One laboratory (Table 5-2). Casein content and yield were not affected by treatment ($P > 0.10$) and casein expressed as a percent of true protein was also similar between the control and L treatments. Despite the limited replication in this study, NCN was 7.4% higher during the L treatment ($P = 0.006$). In a larger production study discussed in Chapter 4, treatment with L at a higher dose (0.2 IU/kg BW, twice daily) also increased the amount of NCN in milk protein, similar to that reported here. In that study, cows treated with L also had higher casein content and yield than control cows. Mackle et al. (1999b) also observed an increase in casein and NCN under conditions of elevated circulating insulin. Similarly, Molento and coworkers (2002) observed an increase in casein percent under a 60% increase in circulating insulin concentrations, though casein and protein yields themselves were not different in that study. The lack of a casein and overall true protein response in

Table 5-3. *Composition of milk proteins as affected by twice-daily subcutaneous injections of insulin glargine in lactating dairy cows*

	Treatment[1]			*P*-value
Variable	Control	Insulin glargine	SEM	Trt
Crude protein,[2] %	3.12	3.27	0.19	0.38
True protein,[3] %	2.93	3.08	0.19	0.37
Casein,[4] %	2.38	2.47	0.12	0.51
Casein, kg/d	1.12	1.13	0.16	0.93
Casein, % of true protein	81.14	80.31	1.24	0.34
Whey,[5] %	0.55	0.61	0.07	0.012
Whey, kg/d	0.26	0.28	0.06	0.015
NCN x 6.38,[6] %	0.74	0.79	0.07	0.006
NPN x 6.38,[6] %	0.19	0.19	0.00	0.49
Milk urea nitrogen,[7] mg/dl	14.1	14.3	1.63	0.92

[1]During control treatment, cows received twice-daily subcutaneous injections of 2 ml of sterile water. During insulin glargine treatment, cows received twice-daily subcutaneous injections of insulin glargine (0.15 IU/kg BW), for a total daily dose of 0.30 IU/kg.
[2] Crude protein is equal to total nitrogen (TN) x 6.38.
[3] True protein (TP) calculated as (TN - NPN) x 6.38.
[4] Casein protein is calculated as (TN - NCN) x 6.38.
[5] Whey protein calculated as (NCN - NPN) x 6.38.
[6] Both NPN and NCN are multiplied by 6.38 to allow comparison with other protein fractions.
[7] Milk urea nitrogen was measured using an infrared method.

the present study may be explained by the elevated SCC observed for one cow in this small-scale experiment, as high SCC can increase proteolysis of casein in raw milk (Verdi et al., 1987; Ma et al., 2000).

Mammary Blood Flow

Estimated mammary blood flow was not affected by treatment with L (P = 0.33). The least squares means for mammary blood flow were 725 and 627 mls blood/ml milk (± 135) for the control and L treatments, respectively. On a per hour basis, estimated mammary blood flow was 1398 and 1172 L/hr (± 126.6) for the control and L treatment periods, respectively (P = 0.26). Treatment with insulin has been reported to have varied results on mammary blood flow in ruminants. Infusion of insulin into the external pudic artery, without glucose infusion, decreased blood flow by 30% in lactating cows (Metcalf et al., 1991). Hyperinsulinemic-euglycemic clamps appear to increase blood flow when compared to control animals (Mackle et al., 2000;

Bequette et al., 2001; Bequette et al., 2002). The disparity between an increase or decrease in blood flow appears to be dependent on co-infusion of glucose to maintain euglycemia. Bequette et al. (2001) used ultrasonic flow probes to measure mammary blood flow during hyperinsulinemia in goats and observed that when blood glucose concentrations fell below normal concentrations, blood flow was also lower and as euglycemia was eventually established through glucose infusion, blood flow increased to higher levels. Duodenal infusion of glucose at increasing doses caused linear increases in mammary blood flow coinciding with a linear increase in plasma glucose concentrations (Rulquin et al., 2004). However, close arterial infusion of glucose to the mammary gland blood supply resulted in a decrease in blood flow (Cant et al., 2002), suggesting that whole-body glucose metabolism may have different effects on blood flow than glucose supply available to the mammary gland.

Plasma Hormones and Metabolites

Results for plasma metabolite and hormone analysis are shown in Table 5-4. As expected, both arterial and venous concentrations of plasma glucose were decreased by approximately 10% ($P < 0.001$) during the L treatment period. Glucose concentrations during the L treatment were still within normal physiological ranges for lactating dairy cows and arterial plasma concentrations averaged 63.1 and 56.9 mg/dl for the control and L treatments, respectively. The AV difference for glucose across the mammary gland was also lower during the L treatment period ($P = 0.001$) and when taking blood flow into account, mammary glucose uptake was reduced by 23% during the L treatment ($P < 0.001$). The AV differences observed in the present study were similar to those reported elsewhere (Metcalf et al., 1991; Mackle et al., 2000). However, under conditions of the hyperinsulinemic-euglycemic clamp, uptake of glucose across the mammary gland is commonly higher during the clamp (Mackle et al., 2000), perhaps due to the additional glucose, and thus energy supply, to the animal.

Table 5-4. *Effect of twice-daily subcutaneous injections of insulin glargine in lactating dairy cows on plasma concentrations of glucose, insulin, and urea nitrogen.*

	Treatment[1]			*P*-value
Variable	Control	Insulin glargine	SEM	Trt
Glucose				
Arterial, mg/dl	63.1	56.9	0.77	<0.001
Venous, mg/dl	45.4	40.7	0.54	<0.001
Arteriovenous difference, mg/dl	17.8	16.2	0.93	0.001
Extraction efficiency, %	28.2	28.4	1.22	0.71
Uptake, g/h	247.7	190.6	10.39	<0.001
Plasma urea nitrogen				
Arterial, mg/dl	10.4	10.3	1.06	0.81
Insulin				
Arterial, ng/ml	0.89	1.20	0.11	0.009
Venous, ng/ml	0.85	1.21	0.12	0.002

[1]During control treatment, cows received twice-daily subcutaneous injections of 2 ml of sterile water. During insulin glargine treatment, cows received twice-daily subcutaneous injections of insulin glargine (0.15 IU/kg BW), for a total daily dose of 0.30 IU/kg.

In this study, it would be expected that the decrease in mammary glucose uptake is not related to direct effects of L on the mammary gland, per se. Glucose uptake in the mammary gland is independent of insulin as GLUT 1 acts in an insulin-independent manner. Other studies have shown no effect of insulin on glucose uptake in the mammary gland (Laarveld et al., 1981; Metcalf et al., 1991), suggesting that the changes in blood flow observed in the present study, though not statistically different, were the main explanatory variable for the reduction in glucose uptake during L treatment.

Arterial plasma urea nitrogen (PUN) did not differ by treatment ($P > 0.10$) and averaged slightly more than 14 mg/dl for both treatments. Though studies with hyperinsulinemic-euglycemic clamps have shown reduced PUN during clamp periods (Mackle et al., 1999b; Bequette et al., 2002), in these studies, there were also significant increases in milk protein production by the mammary gland, suggesting increased utilization of nitrogen under those experimental conditions. As there was no change in either true milk protein or milk urea nitrogen in the present study, changes in PUN would not be expected given these observations.

Arterial and venous plasma insulin concentrations were about 30% higher during the L treatment period. Though these concentrations are nowhere near the 400 to 500% elevation in hyperinsulinemic-euglycemic clamps (e.g. McGuire et al., 1995; Griinari et al., 1997; Mackle et al., 1999b), given that glucose concentrations decreased by 10%, we were successfully able to increase insulin-like activity without the negative consequences of severe hypoglycemia observed in other studies when insulin alone was infused (Kronfeld et al., 1963; Schmidt, 1966). Molento et al. (2002) used a hyperinsulinemic-euglycemic clamp in high producing cows (49 kg/d milk production), and elevated circulating insulin concentrations only 60% above the saline infusion comparison. In the study by Molento et al. (2002), there was also no effect of insulin on protein yield, perhaps suggesting that greater elevation of insulin-activity is needed to stimulate greater milk protein synthesis in the mammary gland. It should be noted, however, that measurement of actual insulin activity in cows treated with insulin glargine has been difficult, which has been discussed in more detail in Chapter 3. Therefore, changes in glucose concentrations serve as a limited proxy for overall insulin activity.

Amino Acid Utilization

Arterial concentrations of amino acids are presented in Table 5-5 and were generally not different between the control and L treatments. Plasma Gly ($P = 0.096$) and Ser ($P = 0.075$) concentrations were higher during treatment with L, but no other arterial concentrations of individual amino acids were affected by treatment. Arterial concentrations of nonessential amino acids tended to increase during the L treatment period ($P = 0.15$).

Plasma amino acid AV differences are presented in Table 5-6. For the essential amino acids, treatment with L decreased the AV difference of Arg ($P = 0.011$) and there was a tendency for a decrease in the AV difference for Val ($P = 0.101$), though

Table 5-5. *Effect of twice-daily subcutaneous injections of insulin glargine in lactating dairy cows on arterial concentrations (μM) of AA*

	Treatment[1]			*P*-value
Variable	Control	Insulin glargine	SEM	Trt
Essential AA				
Arg	55.4	54.7	7.33	0.76
Cys	16.9	17.4	0.83	0.30
His	35.6	36.7	3.68	0.59
Ile	78.0	73.4	4.23	0.58
Leu	139.6	126.5	6.76	0.24
Lys	63.0	62.8	3.92	0.93
Met	18.9	20.7	2.29	0.31
Phe	39.5	40.6	0.93	0.57
Thr	71.4	75.0	9.15	0.56
Trp	22.5	22.6	2.51	0.93
Val	240.7	230.0	10.65	0.61
Nonessential AA				
Ala	171.0	173.5	18.39	0.70
Asn	42.1	50.3	13.27	0.28
Asp	8.6	9.1	0.78	0.74
Cit	70.1	71.0	7.13	0.80
Gln	149.2	160.1	4.63	0.35
Glu	35.2	34.0	2.09	0.58
Gly	239.6	265.2	41.31	0.096
Orn	32.5	30.7	5.75	0.74
Pro	64.1	73.9	7.06	0.20
Ser	71.0	80.1	7.10	0.075
Tyr	46.0	45.7	4.97	0.95

[1]During control treatment, cows received twice-daily subcutaneous injections of 2 ml of sterile water. During insulin glargine treatment, cows received twice-daily subcutaneous injections of insulin glargine (0.15 IU/kg BW), for a total daily dose of 0.30 IU/kg.

these differences are quite small numerically. Treatment with L increased or tended to increase the AV differences for Cys ($P = 0.031$), Lys ($P = 0.145$), Phe ($P = 0.089$), and Trp ($P = 0.099$). Of the nonessential amino acids, the AV differences were higher for Asn ($P = 0.050$) and Cit ($P = 0.027$) during the L treatment. The AV difference for Glu was lower ($P = 0.043$) during L treatment.

When the amino acids were aggregated into either essential, non-essential, branched-chain, glucogenic or total amino acid groups (Table 5-7), essential and nonessential amino acid uptake by the mammary gland was reduced by twice-

Table 5-6. *Effect of twice-daily subcutaneous injections of insulin glargine in lactating dairy cows on arteriovenous concentrations differences (μM) of AA*

	Treatment[1]			*P*-value
Variable	Control	Insulin glargine	SEM	Trt
Essential AA				
Arg	30.0	29.2	3.97	0.011
Cys	1.0	1.4	0.19	0.031
His	9.4	9.3	1.24	0.84
Ile	30.2	29.8	2.78	0.18
Leu	52.3	47.0	4.40	0.55
Lys	37.8	39.5	4.05	0.15
Met	9.3	9.4	1.48	0.23
Phe	13.8	15.9	2.19	0.089
Thr	21.2	21.1	2.82	0.94
Trp	1.0	2.3	0.26	0.099
Val	40.9	40.2	3.49	0.101
Nonessential AA				
Ala	37.6	32.3	6.70	0.18
Asn	8.9	14.4	6.62	0.050
Asp	2.4	2.5	0.13	0.67
Cit	4.6	8.0	0.61	0.027
Gln	34.6	36.8	3.50	0.22
Glu	25.7	23.6	1.96	0.043
Gly	1.2	0.3	1.75	0.25
Orn	16.6	15.5	3.72	0.71
Pro	12.2	13.6	1.71	0.64
Ser	22.2	21.9	6.78	0.80
Tyr	14.7	14.8	2.03	0.88

[1]During control treatment, cows received twice-daily subcutaneous injections of 2 ml of sterile water. During insulin glargine treatment, cows received twice-daily subcutaneous injections of insulin glargine (0.15 IU/kg BW), for a total daily dose of 0.30 IU/kg.

daily injections of L ($P = 0.059$ and $P = 0.086$, for essential and nonessential AA, respectively). Uptake of essential amino acids was reduced 16.3% by treatment with L, and 60.1% of the decrease in essential amino acids can be accounted for by the reduction in uptake of the branched-chain amino acids. Uptake of the branched-chain amino acids was reduced 19.7% by treatment with L ($P = 0.031$). Metcalf et al. (1991) also observed a decrease in the uptake of amino acids when insulin was infused into the external pudic artery. The insulin glargine treatment tended to reduce uptake of the glucogenic amino acids ($P = 0.10$), and the uptake to output ratio for the glucogenic

Table 5-7. *Effect of twice-daily subcutaneous injections of insulin glargine in lactating dairy cows on mammary amino acid utilization*

	Treatment[1]			*P*-value
Variable	Control	Insulin glargine	SEM	Trt
Essential AA[2]				
Arterial, μ*M*	781	760	32.9	0.73
Venous, μ*M*	535	515	28.4	0.71
AV difference, μ*M*	247	245	24.0	0.89
Extraction efficiency, %	31.6	32.2	2.77	0.88
Uptake, m*M*/hr	344.0	287.8	3.67	0.059
Output, m*M*/hr	255.6	235.0	13.25	0.29
Uptake:output ratio	1.34	1.23	0.061	0.16
Nonessential AA[3]				
Arterial, μ*M*	929	993	103.6	0.21
Venous, μ*M*	749	810	73.4	0.26
AV difference, μ*M*	180	184	33.5	0.52
Extraction efficiency, %	19.2	18.4	1.61	0.54
Uptake, m*M*/hr	249.9	214.6	21.98	0.086
Output, m*M*/hr	300.7	275.6	15.29	0.24
Uptake:output ratio	0.83	0.78	0.039	0.13
Branched-chain AA				
Arterial, μ*M*	458	430	18.0	0.46
Venous, μ*M*	335	313	25.1	0.65
AV difference, μ*M*	123	117	8.5	0.58
Extraction efficiency, %	27.1	27.2	2.95	0.97
Uptake, m*M*/hr	171.7	137.9	4.23	0.031
Output, m*M*/hr	118.0	108.5	6.13	0.29
Uptake:output ratio	1.45	1.28	0.113	0.17
Glucogenic AA[4]				
Arterial, μ*M*	1020	1085	117.3	0.24
Venous, μ*M*	801	863	79.3	0.28
AV difference, μ*M*	220	222	38.7	0.64
Extraction efficiency, %	21.4	20.4	1.58	0.49
Uptake, m*M*/hr	304.8	259.8	24.30	0.10
Output, m*M*/hr	325.1	299.0	16.57	0.25
Uptake:output ratio	0.93	0.87	0.035	0.052
Total AA				
Arterial, μ*M*	1710	1754	133.4	0.65
Venous, μ*M*	1284	1325	82.9	0.70
AV difference, μ*M*	427	429	57.3	0.93
Extraction efficiency, %	24.9	24.4	1.84	0.84
Uptake, m*M*/hr	594.0	502.4	24.35	0.077
Output, m*M*/hr	556.3	510.5	28.54	0.26
Uptake:output ratio	1.06	0.99	0.011	0.13

[1]During control treatment, cows received twice-daily subcutaneous injections of 2 ml of sterile water. During insulin glargine treatment, cows received twice-daily subcutaneous injections of insulin glargine (0.15 IU/kg BW), for a total daily dose of 0.30 IU/kg.
[2]Essential AA include: Arg, Cys, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val.
[3]Nonessential AA include: Ala, Asn, Asp, Gln, Glu, Gly, Pro, Ser, Orn, Cit, and Tyr.
[4]Glucogenic AA include all NEAA plus Arg and His.

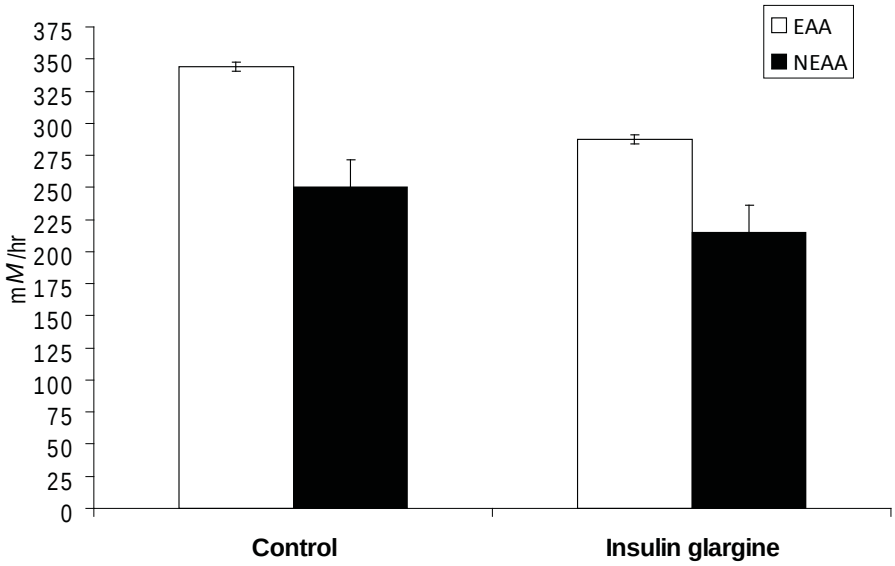

Figure 5-1. *Effects of twice-daily subcutaneous injections of insulin glargine on mammary uptake of essential and nonessential AA (mM/hr). Data presented are least square means (± standard error) for essential (white bars) and non-essential (black bars) amino acids. Cows received either 2 ml of sterile water or 0.15 IU insulin glargine/kg BW twice daily by subcutaneous injection during the treatment periods. Essential and nonessential amino acid uptake by the mammary gland was reduced by insulin glargine treatment (P = 0.059 and P = 0.086, for essential and nonessential AA, respectively).*

amino acids was also reduced by treatment with L ($P = 0.052$). Recall, however, that milk protein yield was not different between treatments (Table 5-2 and Table 5-3). The observation that the mammary gland had reduced total amino acid uptake ($P = 0.077$) yet maintained similar milk protein yield suggests mammary gland utilization of amino acids was altered by treatment with L. Though the total uptake to output of total amino acids was nearly balanced for both treatments, there was a tendency for a difference in the uptake to output ratios ($P = 0.13$, Table 5-7) with the ratio being 1.06 during the conrol treatment and 0.99 during treatment with L. The amino acid balance across the mammary gland is similar to cows subjected to the hyperinsulinemic-euglycemic clamp (Mackle et al., 2000) and generally the amount of amino acid N taken up by the mammary gland can be accounted for in milk protein (Mepham

and Linzell, 1966). In lactating goats, Bequette et al., (2001) also observed reduced mammary uptake of plasma AA under a hyperinsulinemic-euglycemic clamp. In that study the authors suggested that insulin may reduce catabolism of amino acids in the mammary gland, thereby sparing them for milk protein synthesis.

CONCLUSIONS

Though no overall change in either milk protein content or yield was observed in the present study, data from this study suggest that L alters uptake and utilization efficiency of amino acids in the mammary gland. Based on the research reported here and elsewhere, it appears the effects of insulin on amino acid uptake and use by the mammary gland differs by how much insulin is elevated in the cow and by whether glucose is also infused during insulin infusion or treatment.

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CHAPTER SIX: INTEGRATED SUMMARY AND CONCLUSIONS

Most research related to improving milk protein production in lactating dairy cows has focused on determining and meeting the amino acid needs of the cow and using dietary approaches to maximize N efficiency. Despite large improvements in our understanding of the cow's nutrient requirements for optimal milk production, there is still a gap in the knowledge surrounding milk protein synthesis. Rather than looking at milk protein synthesis from a dietary point of view and thinking of amino acids purely as substrates for protein synthesis, employing alternative approaches focused on factors affecting the regulation of milk protein synthesis in the mammary gland have had impressive responses. Alteration of metabolism and hormone concentrations in lactating cows has produced more significant improvements in milk protein yield than dietary approaches. Evidence from hyperinsulinemic-euglycemic clamp studies where milk protein output has increased by up to 28% (Griinari et al., 1997) suggests that there is opportunity for improving milk protein production in the modern dairy cow beyond current management conditions.

Though hyperinsulinemic-euglycemic clamps are useful in an experimental context, the practical application of these studies has not fully been explored. Additionally, during hyperinsulinemic-euglycemic clamps, although insulin is credited as the source of any changes in production or metabolism that occur, significant amounts of glucose, and thus energy, are being infused into the cow. In the study by Mackle (1999), the amount of glucose infused per day (3.336 kg) was the equivalent of 12.2 Mcal NE_L/d (Leonard and Block, 1997), which is a considerable amount of energy. Additionally, intravenous (Amaral et al., 1990) and duodenal (Rulquin et al., 2004) glucose infusions have been shown to increase milk protein yield, so the effects of glucose and insulin are confounded. It would be ideal to evaluate the effects of insulin without concurrent glucose infusion. However, when insulin is administered

alone, a confounding effect of hypoglycemia is also present (Kronfeld et al., 1963; Schmidt, 1966) making research on the effects of insulin a challenge.

Numerous insulins and insulin analogues are available in human medicine for a variety of uses and needs, including insulin products that have extended duration of action and release into the body of the subject. The potential to use slow-release insulin products from human medicine in lactating dairy cows offers opportunity to study the effects of insulin without the need to provide glucose to maintain normal glycemic state.

For this doctoral research, two insulin sources from human medicine were selected based on their prior use in dairy cows (Humulin-N or H; Hayirli et al., 2002) and pharmacokinetic and pharmacodynamic properties (insulin glargine or L). To test the effects of these two forms of slow-release insulin on the metabolism and production of lactating dairy cows, three experiments were carried out and included a dose-response study, a production trial, and a mammary metabolism study.

In Chapter 3, both slow-release insulin sources were used in a dose-response study to evaluate the effects of both insulin types on lactating dairy cow plasma profiles of insulin and glucose. In separate experiments, cows were given a single subcutaneous injection of either H or L at doses of 0, 0.1, 0.2, or 0.4 IU/kg BW. Increasing doses of both H and L caused linear decreases in plasma glucose concentrations in lactating dairy cows over a 24-h period. Plasma insulin concentrations increased with increasing dose of H. Though a standard assay to measure L and its metabolites in plasma is not available, it was evident by the changes in plasma glucose that L exerted insulin-like effects in dairy cows. There were no changes in dry matter intake (DMI) on the day of the injection and sampling, suggesting that critical thresholds for hypoglycemia were not reached with the doses given. However, the highest doses of H and L caused plasma glucose decrease to 20 mg/dl in some cows, which is certainly below normal thresholds for euglycemia

in dairy cows. The objective of the overall research was to determine a dose of both forms of insulin that could be used to study the effects of insulin without large decreases in glycemia. Therefore, it was decided not to use the highest dose of either insulin in a once-daily injection. Based on the 24-h profiles of glucose following H and L insulin injection, it was decided to proceed with intermediate doses (0.15 and 0.2 IU/kg), given twice daily in further experiments.

In Chapter 4, both types of slow-release insulin were used in a study to determine their effects on milk production. Cows were blocked into two groups and within each group randomly assigned to control (C), H, or L treatments. The slow-release insulins were given twice daily at 12-h intervals via subcutaneous injection at a dose rate of 0.2 IU/kg BW, for a total daily dose of 0.4 IU/kg BW. There were no effects of treatment on DMI or overall milk yield. Protein and fat content increased by treatment with both H and L, but the overall yields of fat and protein were only increased by treatment with L. Lactose content decreased for cows treated with H and L, but lactose yield was only lower for L. Milk urea nitrogen concentrations were decreased by treatment with H and L. The conversion efficiency of the predicted MP supply into milk protein observed here suggest that insulin may be altering the N economy of the cow and whole-body metabolism. As expected, plasma glucose concentrations were lower for cows treated with both slow-release insulin products, but the glucose concentrations were still within normal physiological ranges for lactating dairy cows. To explain some of the milk component responses observed here, proteins from mammary biopsies taken at the end of the experiment were analyzed for signaling proteins related to the mammalian target of rapamycin (mTOR) signaling cascade, which is involved with global protein synthesis. The ratio of phosphorylated Akt to total Akt differed by treatment was greatest for H. However, ratio of phosphorylated rpS6 to total rpS6 was not different among treatments. Though there was no difference in the downstream signal of mTOR (rpS6) in the mammary proteins,

the timing of the mammary biopsy may have affected the ability to detect differences in these treatments. Further work will need to be carried out to fully elucidate the cellular signaling by which H and L stimulate milk protein and fat synthesis in high producing cows.

In Chapter 5, a mammary metabolism study was carried out to evaluate the effect of L on amino acid uptake and use by the mammary gland. During the 4-d treatment period, cows were given 0.15 IU/kg BW of L, twice daily at 12-h intervals, for a total daily dose of 0.3 IU/kg BW. Simultaneous blood samples taken from the intercostal artery and subcutaneous abdominal vein allowed measurement of mammary clearance of amino acids and plasma metabolites. Though milk yield tended to be decreased by L in that study, subclinical mastitis may have been the causative agent explaining that observation. Regardless, milk protein yield was not different, despite the tendency to have lower total milk yield. Uptake of the essential and nonessential amino acids was reduced during treatment with L. The ability of the mammary gland to produce the same amount of milk protein with fewer substrates (amino acids), suggests slightly altered mammary metabolism during treatment with L.

Based on the research presented in this dissertation, it is apparent that insulin affects mammary metabolism and milk component synthesis. This research has shown that the effects of insulin can be studied in dairy cows without glucose infusion through use of slow-release insulin products used in human medicine. Additionally, this research supports the data from the hyperinsulinemic-euglycemic clamp studies (Griinari et al., 1997; Mackle et al., 1999), where impressive increases in milk protein production were observed. Though the increases in protein yield reported in Chapter 4 were much less than those observed in the clamp studies, particularly the clamp studies with supplemental amino acid or casein infusions, this research further defines the role of insulin in milk component production, without the confounding effect of glucose infusion. This research also supports the role for

insulin in changing mammary metabolism and utilization of amino acids, increasing the efficiency of use of amino acids within the gland. Though the molecular mechanisms explaining these observations were not discernable from the mammary biopsies taken 12 to 16 h after the last insulin injection in the production study, mTOR signaling is still strongly hypothesized to be the signaling cascade induced by the insulin treatments described herein. The next steps for this line of research may include further defining the molecular mechanism by which H and L alter mammary metabolism to increase milk component production, evaluating longer-term effects of administration of H or L in dairy cows, looking at effects of H and L over various stages of lactation in lactating cows, and exploring dietary strategies to alter endogenous insulin production by the cow.

The potential to make adjustments in diet to alter endogenous insulin secretion have been demonstrated previously in cows. Low forage to concentrate ratios in diets can increase insulin release in dairy cows (Jenny and Polan, 1975). In early lactation cows, plasma insulin concentrations were 52 and 41% higher on an insulinogenic diet for cows of high and low genetic merit than counterparts fed a control diet (Gong et al., 2002). In another early lactation study, cows fed a high starch diet had linear increase in plasma insulin concentrations when starch replaced fat in the diet (Garnsworthy et al., 2008). In the study by Garnsworthy et al., (2008) there were no differences among body condition scores during the 70 d experiment. Based on these observations, endogenous insulin secretion can be altered in dairy cows with changes in diet. However, the more important questions are: to what extent can endogenous insulin synthesis be increased to gain the potential benefits of improved milk protein production, and what are other potential consequences of altering a cow's insulin production by dietary manipulation over the long term?

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APPENDIX

The images below are the scans obtained from developed film from Western blot analysis described in Chapter 4 for phosphorylated ratios of Akt and rpS6. The lanes are labeled with the cow number and treatment and this orientation is the same for all blots.